Analytical and Toxicological Analysis of Perfluorinated Compounds Present in the Environment

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ABSTRACT

Perfluorinated compounds (PFCs) have been produced in relatively large quantities since the 1950s for a wide range of applications such as carpet coatings, food packaging, shampoos, paper, and fire-fighting foams. PFCs are globally ubiquitous in both remote and urban environments. PFCs are present in various matrices including; human blood (whole, plasma and serum), sediments, water, and wildlife. The primary objective of the research described herein is to address data-gaps which have hindered attempts to effectively characterize the risks of PFCs to both humans and wildlife. The hypotheses of this thesis are that (1) current analytical methods for the detection of PFCs are flawed and are in need of improvement, (2) concentrations of PFCs in South Korea are elevated and there is associated risk to both humans and wildlife, and (3) model PFCs such as perfluorooctanesulfonate (PFOS) and perfluorooctanoic acid (PFOA) are poor predictors of effects caused by exposure to other PFCs.

Accurate and precise measurement is essential for effective decision making regarding the production and usage of PFCs. Many issues such as impure standards (either homologs of the standards or unrelated compounds), interactions between isotopically labeled and non-labeled analytes, and the presence of multiple isomers complicate the accurate and precise quantification of PFCs. It has been reported that the relative response factors of isotopically labeled standards and unlabeled standards of the same PFC could be different. Individual (100 ng mL^{-1}) solutions of PFOA and PFOS were analyzed using HPLC-MS/MS under negative-ion-electrospray to detect any impurities present down to 0.5 to 0.1% relative to the major component. Purity of the standards ranged from approximately 86% to ≥ 97%. Standard solutions of unlabeled and isotopically labeled materials were analyzed to compare response factors of isotopically labeled analytes versus their non-labeled counterparts in three different matrices at equivalent concentrations: organic solvent (methanol), serum extract, and water present individually and

concurrently. Not all labeled analytes have the same response factor as their non-labeled complement, and in at least one case the matrix in which the standard is present may cause significant suppression of response. Standard solutions of electrochemical fluorination produced PFOA and PFOS were quantified under multiple reaction monitoring (MRM) mode, using calibration curves prepared from standards consisting primarily of linear standards only, and our results have shown that in general, the use of linear only standards may cause under prediction of concentrations, and that the working range of these standards may be limited.

Previous studies have reported concentrations of PFCs in Asia to be relatively great and in particular Korea has shown to have some of the highest environmental levels ever detected. Despite this fact, relatively little was known about sources, distribution and fate among matrixes. In 2008 and 2009 soil, sediment, water, and biota were collected from the western coast of Korea and were analyzed to determine occurrence and sources of PFCs. PFCs were significantly concentrated in some water and biological samples, while concentrations of PFCs in soils and sediments were relatively low. The most widely detected compound was found to be PFOS, with maximum in water of 450 ng/L and in fish of 612 ng/g, dw. PFOS in water and biota were both less than those thought to cause toxicity, however; in both cases concentrations were within a factor of 10 to a possible toxicity threshold concentration. Although in general the concentrations of PFCs in all three media were reduced from 2008 levels, the calculated bioconcentration factor for PFOS in fish was among the highest ever reported. Overall, the detection of PFCs at relatively great concentrations in various environmental matrices from this region of Korea suggests that further study and characterization of these chemicals and their potential risk to both humans and wildlife is needed.

While PFOS has been extensively studied, other PFCs including replacement chemicals such as perfluorobutanesulfonate (PFBS) and perfluorobutyrate (PFBA), have not been well characterized. Despite the relative lack of data available describing these other PFCs it has been assumed that they will cause similar or lesser effects than PFOS. To test the null hypothesis that all PFCs, including shorter chain-length replacements, act via similar modes of action, rat H4IIE cells were exposed to 10 PFCs, all of which are routinely found in the environment, and the mRNA abundance of 7 target genes was quantified using real-time PCR. Significant changes in mRNA abundance were observed. Effects caused by the shorter chain replacement chemicals differed significantly from those caused by PFOS or PFOA. Furthermore, not all of the PFCs caused the same effects, and changes could not simply be attributed to chain-length or functional group. These differences support that these replacement chemicals do not act through the same mechanisms as the more studied PFOS and PFOA.

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LIST OF ABBREVIATIONS

Koc	Adsorption coefficient
ANOVA	Analysis of variance
ApoA4	Apolipoprotein A-IV
AU	Arbitrary units
AWV	Avian wildlife value
BAF	Bioaccumulation factor
BCF	Bioconcentration factors
CCC	Criteria continuous concentration
CMC	Criteria maximum concentration
Kdes	Desorption coefficient
Kd	Distribution coefficient
ECF	Electrochemical fluorination
ESI-MS/MS	Electrospray-ionization tandem mass spectrometry
FBS	Fetal bovine serum
FAV	Final acute value
FCs	Fluorinated compounds
G-3-PDH	Glyceraldehyde-3-phosphate dehydrogenase
GLI	Great Lakes Initiative
HPLC	High performance liquid chromatography
(Hex)	Homeobox
BCFK	Kinetic bioconcentration factor
LC	Lethal concentration
LOD	Limit of detection
LOD	Limit of detection

LOQ	Limit of quantification
LOEC	Lowest observable effect concentration
MS	Mass spectrometry
Mito-3-Keto-α	Mitochondria 3-ketoacyl-CoA thiolase
Mito-3-Keto-β	Mitochondria 3-ketoacyl-CoA thiolase
MRM	Multiple reaction monitoring mode
NOAEC	No observable adverse effect concentration
NOEC	No observable effect concentration
NMR	Nuclear magnetic resonance
OECD	Organisation for Economic Co-operation and Development
Pax 8	Paired box gene 8
PFBA	Perfluorbutanoic acid
PFCs	Perfluorinated compounds
PFFAs	Perfluorinated fatty acids
PFBS	Perfluorobutanesulfonate
PFDS	Perfluorodecanesulfonate
PFDoA	Perfluorodecanoic acid
PFDA	Perfluorodecanoic acid
PFHpA	Perfluoroheptanoic acid
PFHxS	Perfluorohexanesulfonate
PFHxA	Perfluorohexanoic acid
PFNA	Perfluorononanoic acid
PFOS	Perfluorooctanesulfonate
PFOA	Perfluorooctanoate or Perfluorooctanoic Acid
PFPnA	Perfluoropentanoic acid

PFTeDA	Perfluorotetradecanoic acid
PFTrDA	Perfluorotridecanoic acid
PFUnA	Perfluoroundecanoic acid
Per-3-Keto-α	Peroxisome 3-ketoacyl-CoA thiolase
PCBs	Polychlorinated biphenyls
PP	Polyporopylene
PTFE	Polytetrafluoroethylene
PAPS	Ppolyfluoroalkyl phosphate esters
qPCR	Quantitative real-time PCR
QSAR	Quantitative structure activity relationship
RT-PCR	Real-time polymerase chain reaction
SPE	Solid phase extraction
Sqsyn	Squalene synthase
TRVs	Toxicity threshold values

1 INTRODUCTION

Perfluorinated compounds are a recently discovered, widely distributed class of persistent organic pollutants that have been produced in relatively large quantities since the 1950s for a wide range of applications such as carpet coatings, food packaging, shampoos, paper, and fire-fighting foams. Because of their recent discovery and their unique physical-chemical properties, many analytical, environmental, and toxicological questions still remain unanswered. This thesis will answer some of these questions and to bridge the knowledge gaps that are currently preventing reliable environmental risk assessment.

Although PFCs have been produced on a large scale for more than 40 yr, it was not until the late 1990's that researchers started detecting PFCs in the environment. This was due to a number of factors including: lack of accurate and efficient methods for extraction, lack of standards, especially isotopically labeled ones, and lack of instrumentation with sufficient sensitivity. Accurate and precise measurement is essential for effective decision making regarding the production and usage of PFCs, yet even with the advent of new technology, many issues still remain such as impure standards (either homologs of the standards or unrelated compounds), interactions between isotopically labeled and non-labeled analytes, and the presence of multiple isomers complicate the accurate and precise quantification of PFCs. Therefore the first principal goal of this thesis is to improve current analytical methods for the detection of PFCs in a wide range of matrices.

Previous studies have reported concentrations of PFCs in Asia to be relatively great and in particular, studies have shown Korea to have some of the highest environmental levels ever detected. Despite this fact, relatively little was known about sources, distribution and fate among matrixes; including sediment, soil, water, and biota, and what risk these elevated concentrations

may pose to both humans and wildlife in the region. To this end, the next goal of my thesis is to determine the extent of PFC pollution in South Korea and also to determine the associated risks for both humans and wildlife are.

While PFOS and PFOA have been extensively studied, many other PFCs, and in particular shorter chain replacement chemicals, have not been well characterized. Despite this poor characterization, it has been assumed that these other commonly detected PFCs will cause similar or lesser effects compared to PFOS or PFOA. Hence, the third goal of my thesis was designed to compare other commonly detected PFCs, to model PFCs such as PFOS and PFOA, and to determine whether they were good predictors of the effects caused by exposure to other PFCs.

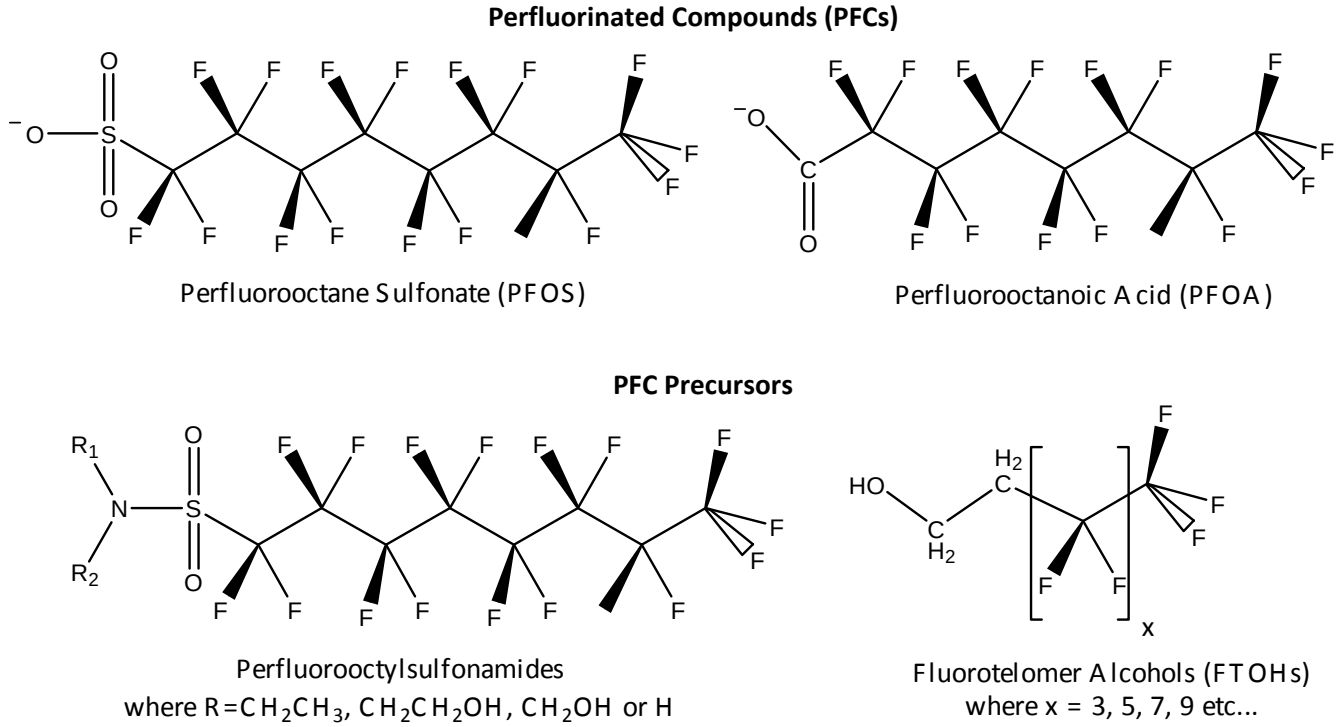
Some degree of redundancy in the presentation of this PhD thesis was unavoidable as each chapter was written independently for publication in peer-reviewed journals. At the time of submission of this PhD thesis, Chapters 3 and 4 were previously published in the academic journals Toxicological and Environmental Chemistry and Environmental Pollution respectively, and chapter 6 has been accepted for publication in the journal Chemoshpere.

2 LITERATURE REVIEW

2.1 Introduction

Perfluorinated compounds are fluorinated at all of the valence electrons of the carbon atoms in organic molecules, or at least a portion of the molecule is perfluorinated (Figure 2.1). All PFCs are synthetic and many have been used in commercially available products or released as byproducts. A partial list of the compounds that are known to have been manufactured and or released into the environment is provided (Table 2.1). These compounds vary in structure, and thus exhibit different environmental fates and toxicities. Unfortunately, there is presently little information describing the chemical-physical properties of most PFCs, and even less toxicity information is available regarding these compounds. There is some information available on the mechanisms of toxic action and acute and chronic toxicity for a few compounds. Most such information is for the two primary PFCs that have been found as residues in the environment: perfluorooctanesulfonate (PFOS) and perfluorooctanoate (PFOA).

Figure 2.1 Structure of perfluorinated compounds and some of their precursors



Among the more predominant PFCs that have been used in the production of commercial or industrial products, and released into the environment, are the perfluorinated fatty acids (PFFAs). The PFFAs are synthetic, fully fluorinated, fatty acid analogues that are characterized by a perfluoro-alkyl chain and a terminal sulfonate or carboxylate group. The high-energy carbon-fluorine (C-F) bond renders these compounds resistant to hydrolysis, photolysis, microbial degradation, and metabolism by animals, which makes them environmentally persistent (Giesy and Kannan 2002). PFCs have been manufactured for over 50 yr and have been used in materials such as wetting agents, lubricants, corrosion inhibitors, stain resistant treatments for leather, paper and clothing, and in foam fire extinguishers (Sohlenius et al. 1994). The global environmental distribution, bioaccumulation, and biomagnification of several

perfluoro-compounds have recently been studied (Giesy and Kannan 2001). PFOS is the predominant perfluorinated compound found in the tissues of wildlife.

Since PFFAs are chemically stabilized by strong covalent C-F bonds, they were historically considered to be metabolically inert and non-toxic (Sargent and Seffl 1970). Accumulating evidence has demonstrated that PFFAs are actually biologically active and can cause peroxisomal proliferation, increased activity of lipid and xenobiotic metabolizing enzymes, and alterations in other important biochemical processes in exposed organisms (Obourn et al. 1997; Sohlenius et al. 1994). In wildlife, the most widely distributed PFFA, PFOS, accumulates primarily in the blood and in liver tissue (Kannan et al. 2002a;Kannan et al. 2001a). Therefore, the major target organ for PFFAs is presumed to be the liver. However, this does not exclude other possible target organs such as the pancreas, testis, and kidney (Olson and Andersen 1983). Until recently, most toxicological studies have been conducted on PFOA and perfluorodecanoic acid (PFDA), rather than on the more environmentally prevalent PFOS. However, PFOS appears to be the ultimate degradation product of several commercially used perfluorinated compounds. Concentrations of PFOS found in wildlife are greater than those of other PFCs (Giesy and Kannan 2002; Kannan et al. 2001a; Kannan et al. 2001b).

A large body of ecotoxicological information, generated over a period of more than 10 yr, exists for various salts of PFOS. However, until recently, definitive information was not available on chemical purity. Validated analytical methodology did not exist to measure exposure concentrations in many of the early studies. Therefore, data generated prior to 1998 were less reliable as to the nature of substance(s) tested, and exposure concentrations were not measured as part of these studies. Therefore, data generated prior to 1998 were less reliable as to

Table 2.1 Perfluorinated compounds (PFCs) and their precursor molecules

Compound (Synonyms)	CAS Number	Molecular Structure	Molecular Wt
PFCs			
Perfluorobutanesulfonate (C4, PFBS)	29420-49-3	$C_4F_9SO_3^-$	299
Perfluorohexanesulfonate (C6, PFHxS)	432-50-7	$C_6F_{13}SO_3^-$	399
Perfluorooctanesulfonate (C8, PFOS)	2795-39-3	$C_8F_{17}SO_3^-$	499
Perfluorooctanesulfonic acid	1763-23-1	$C_8F_{17}SO_3H$	500
Tridecafluoroheptanoate (C7, PFHpA)	-	$C_6F_{13}COO^-$	363
Perfluoroheptanoic acid	375-85-9	$C_6F_{13}COOH$	364
Perfluorooctanoate (C8, PFOA)	-	$C_7F_{15}COO^-$	413
Perfluorooctanoic acid	335-67-1	$C_7F_{15}COOH$	414
Heptadecafluorononoate (C9, PFNA)	-	$C_8F_{17}COO^-$	463
Perfluorononanoic acid	375-95-1	$C_8F_{17}COOH$	464
Nonadecafluorodecanoate (C10, PFDA)	-	$C_9F_{19}COO^-$	513
Perfluorodecanoic acid	335-76-2	$C_9F_{19}COOH$	514
Perfluoroundecanoate (C11, PFUnA)	-	$C_{10}F_{21}COO^-$	563
Perfluoroundecanoic acid	2058-94-8	$C_{10}F_{21}COOH$	564
Perfluorododecanoate (C12, PFDoA)	-	$C_{11}F_{23}COO^-$	613
Perfluorododecanoic acid	307-55-1	$C_{11}F_{23}COOH$	614

Perfluorotridecanoate (C13, PFTrA)	-	$C_{12}F_{25}COO^-$	663
Perfluorotetradecanoate (C14, PFTA)	-	$C_{13}F_{27}COO^-$	713
Perfluorotetradecanoic acid	376-06-7	$C_{13}F_{27}COOH$	714
Perfluoropentadecanoate (C15, PFPA)	-	$C_{14}F_{25}COO^-$	763
PFC-Precursors			
Perfluorooctane sulfonamide (PFOSA)	4151-50-2	$C_8F_{17}SO_2NH_2$	499
n-Methyl perfluorooctane sulfonamidoethanol (n-MeFOSE)	24448-09-7	$C_8F_{17}SO_2N(CH_3)C_2H_4OH$	557
n-Ethyl perfluorooctane sulfonamidoethanol (n-EtFOSE)	1691-99-2	$C_8F_{17}SO_2N(C_2H_5)C_2H_4OH$	571
n-Ethyl perfluorooctane sulfonamidoacetic acid (PFOSAA)	2991-51-7	$C_8F_{17}SO_2N(C_2H_5)CH_2CO_2H$	585
n-Ethyl perfluorooctane sulfonamide (n-EtFOSA)	4151-50-2	$C_8F_{17}SO_2NH(C_2H_5)$	
Perfluorooctane sulfonylfluoride (POSF)	307-35-7	$C_8F_{17}SO_2F$	502
6:2 Fluorotelomer alcohol (6:2 FTOH)	647-42-7	$CF_3(CF_2)_5C_2H_4OH$	364
8:2 Fluorotelomer alcohol (8:2 FTOH)	865-86-1	$CF_3(CF_2)_7C_2H_4OH$	464
10:2 Fluorotelomer alcohol (10:2 FTOH)	678-39-7	$CF_3(CF_2)_9C_2H_4OH$	564

the nature of substance(s) tested, and exposure concentrations were not measured as part of these studies. The potassium salt of PFOS was chosen for many of the laboratory studies that have been cited in this review, because it is the most prominent of all the PFOS salts produced. The commercially prepared potassium product was available as a full-strength salt. For example, in 1997, PFOS-K^+ accounted for >45% of all PFOS salts produced (US EPA 2001). Although the lithium, ammonium, diethanolamine and didecyldimethylammonium salts have been tested, many of the studies on these salts utilized mixtures containing only 25-35% active ingredients. The majority of these studies were conducted in accordance with US EPA and/or OECD Good Laboratory Practices. Older studies have also been included where more recently generated data were not available for various species. In addition, in this assessment I also examine recent studies published in the open literature that pertain to ecological presence and biochemical modes of action of PFFAs.

2.2 Environmental Fate

2.2.1 Physical/Chemical Properties

PFOS is moderately water soluble, non-volatile, and thermally stable. The potassium salt of PFOS has a reported mean solubility of 680 mg/L in pure water. However, PFOS is a strong acid, and in water at a neutral pH it will completely dissociate into the ionic form. Thus, the PFOS anion can form strong ion pairs with many cations, which results in salting out in natural waters that contain relatively great amounts of dissolved solids (Table 2.2). For example, as salt content increases, the solubility of PFOS decreases such that PFOS solubility in sea water is approximately 12.4 mg PFOS/L whereas the solubility is 680 mg PFOS/L in pure water. PFOS has a reported mean solubility of 56 mg PFOS/L in pure octanol. However, due to the surface-active properties of PFOS, when it is added to an octanol/water mixture in a standard test system

to measure K_{ow}, it forms three layers. Thus, an octanol/water partition coefficient has not been directly measured for PFOS, but has been estimated from its water and octanol solubilities. Other physiochemical properties for this molecule such as the bioconcentration factor and the soil adsorption coefficient cannot be estimated with conventional Quantitative Structure Activity Relationship (QSAR) models. The use of K_{ow} is not appropriate to predict these other properties because PFOS does not partition into lipids, but instead binds to proteins such as albumin (Jones et al. 2003). As a result, use of either water solubility or predicted K_{ow} values may underestimate the accumulation of PFOS into organisms and other environmental media. PFOS is not expected to volatilize, based on its vapor pressure and predicted Henry's Law Constant. (OECD et al. 2002) classified PFOS as a type 2, non-volatile chemical that has a very low volatility. Available physical/chemical properties for the potassium salt of PFOS are presented in Table 2.2.

2.2.2 Photolysis

No experimental evidence of direct or indirect photolysis of PFOS is available (Hatfield 2001a). The indirect photolytic half-life for PFOS, using an iron oxide photo-initiator matrix model, was estimated to be $\geq$3.7 yr at 25 ^{o}C. This model was chosen because it minimized the experimental error in this matrix. This half-life is based on the analytical method of detection.

Table 2.2 Physical/chemical properties of the potassium salt of PFOS

Parameter	Value	Reference
Melting Point	≥ 400 °C	Jacobs and Nixon 1999
Boiling Point	Not calculable	OECD 2002
Specific gravity [a]	~ 0.6 (7-8)	OECD 2002
Vapor Pressure	3.31 x10^{-4} Pa @ 20 °C	Van Hoven et al. 1999
Water Solubility		
Pure water	680 mg/L	Ellefson 2001c
Fresh water	370 mg/L	OECD 2002
Sea water	12.4 mg/L	Ellefson 2001a
Octanol Solubility	56 mg/L	Ellefson 2001b
Log K_{ow} [b]	-1.08	OECD 2002
Henry's Law Constant [c]	4.34 x 10^{-7}	OECD 2002

[a] pH values in parentheses

[b] Log K_{ow} calculated from PFOS solubility in water and n-octanol

[c] Henry's Law constant calculated at 20 °C using solubility in pure water

2.2.3 Hydrolysis

Under experimental conditions (50 °C and pH conditions of 1.5, 5, 7, 9, or 11) no hydrolytic loss of PFOS was observed in a 49-d study (Hatfield 2001b). Based on mean values and precision measures, the hydrolytic half-life of PFOS was estimated to be ≥41 yr at 25 °C. However, it is important to note that this estimate was influenced by the analytical limit of quantification, and no loss of PFOS was detected in the study.

2.2.4 Biodegradation

Biodegradation studies in which PFOS was monitored analytically for loss of parent compound have been conducted using a variety of microbial sources and exposure regimes (Lange 2001; Gledhill and Markley 2000a,b,c). In one study with activated sludge, no loss or biotransformation of PFOS was observed over a 20-wk period under aerobic conditions, nor were there any losses observed in a study conducted for 56 d with activated sludge under anaerobic conditions. Findings from these studies are supported by the results from a MITI-I test (Kurume Laboratory 2002) that showed no biodegradation of PFOS after 28 d, as measured by net oxygen demand, loss of total organic carbon or loss of parent material. In addition, no losses of PFOS were observed in a biodegradation study conducted with soil under aerobic conditions. Therefore, to date, no laboratory data exist that demonstrates PFOS undergoes significant biodegradation under environmental conditions.

2.2.5 Thermal Stability

Several studies suggest that PFOS would have relatively low thermal stability. This conclusion is based on the fact that the carbon-sulfur (C-S) bond energy is much weaker than the carbon-carbon (C-C), or the carbon-fluorine (C-F) bond energies, and as a result, would more easily break under incineration conditions (Dixon 2001). This conclusion is supported by a study by Yamada and Taylor (2003) indicating that PFOS should be nearly completely destroyed when incinerated.

2.2.6 Adsorption/Desorption

PFOS appears to adsorb strongly to soil, sediment, and sludge (Table 2.3) with an average distribution coefficient (K_d) greater than 1 ml/g, and an organic carbon normalized adsorption coefficient (K_{oc}) greater than 10,000 ml/g (Ellefson 2001d). Based on these values, PFOS would not be classified as qualitatively mobile, as defined by OECD guidelines. Once adsorbed to these matrices, PFOS does not readily desorb, even when extracted with an organic solvent. The average desorption coefficient (K_{des}) for soils was determined to be less than 0.001 ml/g. In these matrices, adsorption and desorption equilibria were achieved in less than 24 hr; moreover, in more than 50% of cases, equilibria were achieved after approximately 1 min of contact with the test adsorbents. As a result, PFOS exhibited little mobility in all matrices tested, and would not be expected to migrate any significant distance. The shape of the adsorption isotherm (H-type) indicates a very strong chemical/adsorption interaction. Since PFOS is a strong acid, it probably forms strong bonds in soils, sediments, and sludge via a chemisorption mechanism.

2.2.7 Bioconcentration

The potential of PFOS to bioaccumulate and bioconcentrate into fish, and the relative importance of dietary and water-borne sources of PFOS to fish accumulation have been evaluated. In a bioaccumulation study with juvenile rainbow trout (*Oncorhynchus mykiss*), fish were exposed to 0.54 μg PFOS/g in the diet for 34 d, followed by a 41-d depuration phase (Martin et al. 2003b). PFOS was accumulated in and depurated from the liver and carcass in a time-dependent manner. The predicted time to reach 90% steady state would be 43 d, which was approximately the same as the exposure duration in the study. The liver and carcass depuration

Table 2.3 Adsorption and desorption of PFOS to sediments and soils [a]

Soil Type	Adsorption Kinetics			Desorption Kinetics	
	K_d (L/g)	K_{oc} (L/g)	K_{adsF} [b]	K_{des} (L/g)	K_{desF} [b]
Clay	0.0183	70.4	0.0560	0.000471	0.222
Clay Loam	0.00972	37.4	0.0421	0.0000158	0.082
Sandy Loam	0.0353	126	0.0919	0.0000349	0.104
River Sediment	0.00742	57.1	0.0094	0.0000100	0.039
Domestic Sludge	<0.120	NC [c]	0.0568	<0.000237	29.5

[a] Values of K_d, K_{oc} and K_{des} are averaged values

[b] Freundlich coefficient

[c] NC = not calculable

rate constants were 0.035- and 0.054-d^{-1}, representing depuration half-lives of 20 and 13 d, respectively. The assimilation efficiency was 120 ± 7.9%, which indicates efficient absorption of PFOS from ingested food. This assimilation efficiency is greater than that observed with chlorinated contaminants such as polychlorinated biphenyls (PCBs), where efficiencies in trout can range from 20 to 60% (Fisk et al. 1998). In addition, this assimilation efficiency of PFOS is indicative of entero-hepatic recirculation, which could affect the disposition of PFOS in fish. Evidence of entero-hepatic recirculation in rats has been shown to affect the rate of elimination (Johnson et al. 1984). As a result, this process may also be an important mechanism that helps maintain PFOS concentrations in fish beyond what is predicted from K_{ow} or water solubility values. The bioaccumulation factor (BAF) for PFOS was 0.32 ± 0.05, which indicates that dietary exposure did not result in biomagnification in trout. This small BAF probably resulted from several factors, including a relatively low experimental feeding rate (F=1.5% body wt) coupled with a relatively rapid rate of depuration. Taken together, these data show that under these experimental conditions, the diet would not be a major route of PFOS exposure for fish.

Studies conducted with other fish species have shown that PFOS will bioconcentrate in tissues from waterborne exposures (Table 2.4). Bluegill exposed to 0.086 or 0.87 mg PFOS/L in a flow-through system accumulated PFOS into edible and nonedible (fins, head, and viscera) tissues in a time-dependent manner (Drottar et al. 2001). In this bluegill study, fish were exposed to 0.086 mg PFOS/L for 62 d, but were only exposed to 0.87 mg PFOS/L for 35 d, because of excessive mortality. At the end of the exposure phase of both treatments, PFOS tissue concentrations appeared to still be increasing. As a result, kinetic analyses of the data were conducted to calculate the kinetic bioconcentration factor (BCF_K) from estimated uptake and depuration rate constants. Fish exposed to 0.87 mg PFOS/L were not used to estimate these

parameters. The BCF_K values for edible, inedible and whole fish tissues were calculated to be 1,866, 4,312, and 3,614, respectively. During the elimination phase of the study, PFOS depurated slowly and the time to reach 50% clearance for edible, non-edible and whole fish tissues were 146, 133 and, 152 d, respectively.

Tissue distribution and accumulation kinetics were determined in rainbow trout exposed to 0.35 μg PFOS/L (Martin et al. 2003a). The magnitude of PFOS concentrations in tissues were in the order of blood> kidney> liver> gall bladder. The least concentrations of PFOS were observed in the gonads, followed by adipose, and then muscle tissue (Table 2.4). In blood, approximately 94-99% of the PFOS was associated with plasma, and a minor amount was associated with the cellular fraction. PFOS also accumulated in the gills, indicating their importance in the uptake and depuration in trout.

In general, the depuration rate constants determined for carcass, blood and liver showed that PFOS was more rapidly depurated than are some organochlorine contaminants (PCBs and toxaphene), but the rate is slower than that observed for other surfactants (Fisk et al. 1998; Tolls and Sijm 1995). When compared to other surfactants, uptake rate constants were greater than expected and were directly related to greater tissue concentrations (Tolls et al. 1997). BCF_K were 1,100, 4,300, and 5,400 for carcass, blood, and liver, respectively. As was observed for bluegill, steady state PFOS concentrations in tissues were not achieved at the end of the exposure period.

The 12-d accumulation ratios (BCF divided by tissue concentration at the end of the exposure period) for carcass, blood and liver were greater than 600 indicating that the tissue concentrations were far from steady state. However, values of the BCF_K, calculated for rainbow trout, were well within the range of values observed for other species such as bluegill and carp.

Table 2.4 Kinetic parameters and bioconcentration factors (BCF) of PFOS in fish

Species	Tissue	Apparent BCF [a]	***Kinetic Parameters***			
			K_u (L/kg*d)	K_d (1/d)	BCF_K [b] (L/kg)	Half-life (d)
Bluegill	Edible	484	8.9	0.0047	1866	146
	Unnedible	1124	22	0.0052	4312	133
	Whole	856	16	0.0045	3614	152
Rainbow trout	Carcass	-	53	0.048	1100	15
	Blood	-	240	0.057	4300	12
	Liver	-	260	0.050	5400	14

[a] Apparent BCF was calculated as the concentration in fish at the end of the exposure phase divided by the average water concentration

[b] BCFK was estimated as Ku/Kd

In a flow-through bioconcentration study conducted with carp (*Cyprinus carpio*), fish were exposed to 2 or 20 μg PFOS/L, and water and fish tissue samples were collected throughout testing (Kurume laboratory 2001). Upon sampling, fish were separated into parts that included integument (skin except head, scales, fins, alimentary canal, or gills), head, viscera (internal organs except for alimentary canal and liver), liver, and carcass, and then analyzed for concentrations of PFOS. Kinetic analysis was not conducted because the study was not designed to examine uptake from water; rather, BCFs were calculated in all fish tissues at steady state. Steady state was assumed when three or more consecutive sets of tissue PFOS concentrations were not statistically different. In fish exposed for 58 d, the BCFs in carp from the 2 μg PFOS/L treatment ranged from 200 to 1,500. In fish from the 20 μg PFOS/L exposure, BCFs ranged from 210 to 850. PFOS depurated slowly and the time to reach 50% clearance for fish in the 20 μg PFOS/L treatment was 49 d, whereas 152 d was required for fish in the 2 μg PFOS/L treatment to reach 50% clearance.

To date, laboratory studies have demonstrated that PFOS accumulates into fish in a time- and concentration-dependent manner. In addition, these studies suggest that the primary route of accumulation of PFOS into fish is from exposure to aqueous PFOS. Dietary sources of PFOS are secondary and may not significantly enhance the overall accumulation of PFOS by fish. However, what actually happens under natural environmental conditions is yet to be verified. The reason for this is that discrepancies exist between accumulation factors as measured in the laboratory, and those estimated in field studies. For example, bioaccumulation factors calculated from liver and surface water PFOS concentrations ranged from 6,300 to 125,000 in the common shiner (*Notropus cornutus*) collected in a Canadian creek (Moody et al. 2001). In contrast, the bioconcentration factor for rainbow trout, based on liver concentrations was 5,400,

approximately 23-fold less than the maximal value derived in shiners (Martin et al. 2003b). The discrepancy between laboratory and field accumulation values has also been observed for fish collected from Tokyo Bay, Japan (Taniyasu et al. 2003). In that study, PFOS concentrations in fish livers were similar to those observed in the Great Lakes region of the United States and resulted in bioaccumulation factors that ranged from approximately 1,260 to 19,950. Again, the estimated BAFs were often greater than those measured in laboratory studies. In a field study conducted in a reservoir in the Tennessee River, near Decatur Alabama, fish and surface water samples were collected and analyzed for PFOS. Bioconcentration factors from surface water PFOS concentrations and whole body PFOS concentrations in catfish and largemouth bass ranged from 830 to 26,000 (Giesy et al. 2010). Although BCF values determined in the laboratory are within the lower range of these values, they are also approximately 4-fold less than the greater values estimated with fish from this location. The determination of BCF values from field exposures is complicated by the fact that less polar, PFOS-containing compounds could have been accumulated and then degraded to PFOS. Thus, while there is good agreement between the results of laboratory studies, BCFs and BAFs estimated from field data vary greatly, and in many cases exceed values calculated from studies conducted under laboratory conditions. Factors contributing to variation in values of BAF and BCF developed from field observations may include inter-species and sex-dependent variation in accumulation. In addition, dietary sources of PFOS may be more important in the accumulation of PFOS by fish over their life cycle than would be expected based on results from laboratory studies conducted with rainbow trout. Finally, the accumulation of PFOS precursors, and their subsequent biotransformation into PFOS, may also be a contributing factor to the greater than expected PFOS concentrations in fish collected from the field. Overall, additional studies will have to be conducted to evaluate the

relative importance of different accumulation pathways of PFOS by fish populations under natural environmental conditions.

2.3 Ecotoxicology

Recently, the toxicity of several PFFAs has been intensively studied, although most work has been limited to either PFOS or PFOA (Hekster et al. 2003). Among the PFFAs, PFOS is the most commonly found perfluorinated compound in environmental samples; this compound is particularly prevalent in the tissues of aquatic organisms (Giesy and Kannan 2001). The finding of such residues, in recent years, has resulted in primary efforts to investigate the toxicity of PFOS to aquatic organisms. From laboratory toxicity studies, the PFOS is known to be moderately acutely and slightly chronically toxic to aquatic organisms, in general. In this section, the acute and chronic toxicity of PFOS to aquatic organisms, both for freshwater and marine species, is reviewed.

In 2000, productions of PFOS-based products, or those compounds that can degrade to PFOS, were discontinued by the 3M Company. This was done, in part, because it was possible to substitute the less accumulative and less toxic PFFA, perfluorobutanesulfonate (PFBS). Although PFBS is a widely used replacement for PFFA in many products, few studies have thoroughly studied it and there is considerably less toxicological information available.

2.3.1 Acute Toxicity of PFOS to Aquatic Organisms

Aquatic Macrophytes: Data describing the acute toxicity of PFOS to aquatic plants are somewhat limited (Table 2.5). The acute toxicity of PFOS to duckweed (*Lemna gibba*) has been reported; the number of fronds or biomass produced during the 7-d exposure served as an index

to relative toxicity. There were two primary conclusions: First, the 7-d IC_{50} was found to be 108 mg PFOS/L, with a 95% confidence interval of 46-144 mg PFOS/L, and a no observable effect concentration (NOEC) of 15 mg PFOS/L, based on frond number (Desjardins et al. 2001c). The sublethal effects noted in *L. gibba,* exposed to concentrations ≥31.9 mg PFOS/L, included root destruction and/or cupping of the plant (fronds) downward (convex) on the water surface. There was a concentration-dependent increase in dead, chlorotic, and necrotic fronds at greater PFOS concentrations (147 and 230 mg PFOS/L). A recovery period was not evaluated in this study. Second, *L. gibba* exposed to PFOS, showed a 7-d IC_{50} of 59 mg PFOS/L (52-60 mg PFOS/L) based on the frond number, and an 7-d IC_{50} of 31 mg PFOS/L (22-36 mg PFOS/L) based on the biomass, wt/wt (Boudreau et al. 2003a). Values, based on frond number and biomass, were 29 and 6.6 mg PFOS/L, respectively. At the greatest concentration tested (160 mg PFOS/L), plant fronds exhibited both high percentages of chlorosis and necrosis.

Invertebrates: Several studies describe the acute toxicity of PFOS with the cladoceran *Daphnia magna* (Table 2.5). *D. magna* is a representative species among the aquatic invertebrates that are commonly used in standardized toxicity testing. In these acute toxicity studies, cladocerans were exposed to various concentrations of PFOS for 48 hr, and survival (mortality) and immobility were used as endpoints to calculate LC_{50} or EC_{50} values. Several earlier studies had reported that the LC_{50} for *D. magna* was 58-67 mg PFOS/L (Robertson 1986; Drottar and Krueger 2000b; Boudreau et al. 2003a). However, water concentrations of PFOS were not verified in these studies. Later in 2000, a similar finding was observed in a separate acute toxicity test with *D. magna*, where the 48-hr LC_{50} was reported to be 61 mg PFOS/L with a 95% confidence interval of 33-91 mg PFOS/L (Drottar and Krueger 2000b). The NOEC, based on survival/immobility, was 32 mg/L in that study.

Table 2.5 Acute toxicity of PFOS to aquatic organisms (95% confidence intervals in parentheses)

Trophic level	Test organism/Species	Test Duration	Endpoint	NOEC (mg/L)	LOEC (mg/L)	$EC_{50}/LC_{50}/IC_{50}$ (mg/L)	Reference
Freshwater							
Macroalgae	*Lemna gibba*	7 d	Frond number	15		108 (46-144)	Desjardins et al. 2001c
		7 d	Frond number	29.2		59.1 (51.5-60.3)	Desjardins et al. 2001c
		7 d	Biomass	6.6		31.1 (22.2-36.1)	Boudreau et al. 2003a
Invertebrate	*Daphnia magna*	48 hr	Survival	33.1 (32.8-34.1)		130 (112-136)	Boudreau et al. 2003a
		48 hr	Immobility	0.8 (0.6-1.3)		67.2 (31.3-88.5)	Boudreau et al. 2003a
		48 hr	Survival/immobility	32		61 (33-91)	Drottar and Krueger 2000b
		48 hr	Survival			58 (46-72)	Robertson 1986
		48 hr	Survival			67 (48-92)	Robertson 1986
		48 hr	2nd generation survival	12			Drottar and Krueger 2000f
	Daphnia pulicaria	48 hr	Survival	46.9 (33.1-65.3)		169 (136-213)	Boudreau et al. 2003a
		48 hr	Immobility	13.6 (2.2-33.1)		134 (103-175)	Boudreau et al. 2003a

	Unio complamatus	96 hr	Survival	20		59 (51-68)	Drottar and Krueger 2000c
Amphibians	*Xenopus laevis*	96 hr	Growth	4.82	7.97	15.6	Palmer and Krueger 2001
Fish	*Pimephales promelas*	96 hr	Survival	3.2	5.4	9.1 (7.7-11)	Drottar and Krueger 2000h
	Oncorhynchus mykiss	96 hr	Survival			7.8 (6.2-9.8)	Robertson 1986
		96 hr	Survival			9.9 (7.5-13.4)	Robertson 1986
		96 hr	Survival	6.3	13.0	22 (18-27)	Palmer et al. 2002a
Marine							
Invertebrate	*Artemia salina*	48 hr	Survival			9.4 (7.4-12.1)	Robertson 1986
		48 hr	Survival			9.4 (7.3-12.2)	Robertson 1986
		48 hr	Survival			8.9 (6.7-11.9)	Robertson 1986
	Mysidopsis bahia	96 hr	Survival	1.1		3.6 (3.0-4.6)	Drottar and Krueger 2000d
		96 hr	2nd generation survival	0.53			Drottar and Krueger 2000g
	Crassostrea virginica	96 hr	Shell growth	1.8		>3.0	Drottar and Krueger 2000e
Fish	*Oncorhynchus mykiss*	96 hr	Survival			13.7 (10.7-17.7)	Robertson 1986
		96 hr	Survival			13.7 (10.7-17.8)	Robertson 1986
	Cyprinodon variegatus	96 hr	Survival	<15		>15	Palmer et al. 2002b

Recently, additional acute toxicity tests with *Daphnia* species have been performed following ASTM guidelines (Boudreau et al. 2003a) (Table 2.5). In these studies, the 48-hr LC_{50} for *D. magna* was determined to be 130 mg PFOS/L, and the 48-hr LC_{50} for *D. pulicaria* was determined to be 169 mg PFOS/L. Based on immobility of the cladocerans, the 48-hr EC_{50} values for *D. magna* and *D. pulicaria* were determined to be 67.2 and 134 mg PFOS/L, respectively. NOEC values for *D. magna* (0.8 mg PFOS/L) and *D. pulicaria* (13.6 mg PFOS/L) significantly differed from each other. Differences between studies in reported LC_{50} and NOEC values for PFOS-exposed *Daphnia* species could result from uncertainty in differentiating between the immobility and lethality endpoint. *D. magna* appeared to be more sensitive than *D. pulicaria* where the endpoint was 48-hr immobility (Boudreau et al. 2003a).

In another acute toxicity test with the freshwater mussel (*Unio complamatus*), mussels were exposed to various concentrations of PFOS for 96 hr (Table 2.5). The 96-hr LC_{50} was determined to be 59 mg PFOS/L (51-68 mg PFOS/L), whereas the 96-hr NOEC, based on mortality, was 20 mg PFOS/L (Drottar and Krueger 2000c). Mussel tissues were analyzed for PFOS content in this study. Chemical analysis of tissue showed that there was no mortality associated with 96-hr PFOS exposure of <7.3 mg/kg, wt/wt. In contrast, 90% mortality was observed in mussels containing >88 mg PFOS/kg, wt/wt after 96 hr of exposure.

In addition to freshwater invertebrate toxicity testing, the PFOS toxicity to marine species has also been evaluated for marine species (Table 2.5). In a series of acute toxicity tests with brine shrimp (*Artemia salina*), the average (n=3) 48-hr LC_{50} was 9.2 ± 0.29 mg PFOS/L (Robertson, 1986). In an acute toxicity test with the saltwater mysid (*Mysidopsis bahia*), the 96-hr LC_{50} was 3.6 mg PFOS/L, and the NOEC was determined to be 1.1 mg PFOS/L, based on mortality (Drottar and Krueger 2000d). The effect of PFOS exposure on a benthic marine

invertebrate has also been reported. Shell deposition in the eastern oyster (*Crassostrea virginica*) was examined in this study; shell growth was inhibited at a concentration of 1.8 mg PFOS/L, by 20% compared to controls (Drottar and Krueger 2000e). However, an EC_{50} could not be calculated in this study because growth was only inhibited by 28% at the greatest PFOS concentration of 3.0 mg/L tested. In summary, the acute invertebrate toxicity data indicated that, in short term exposures, marine invertebrates are more sensitive to PFOS exposure than are freshwater species.

Amphibians: The developmental effects of PFOS on the African-clawed frog (*Xenopus laevis*) have been investigated by the Frog Embryo Teratogenesis Assay-Xenopus (FETAX) (Palmer and Krueger 2001). In this assay, frog embryos and tadpoles were exposed to various concentrations of PFOS (0.0-24 mg PFOS/L) for 96 hr, and the endpoints of survival, growth and developmental anomalies were examined during early stages of development. Significant mortality occurred at concentrations >14.4 mg PFOS/L and the 96-hr LC_{50} was found to be 14–18 mg PFOS/L, for the three replicate assays. There was a correlation between PFOS exposure and malformations in each of the three assays, and the most commonly observed malformations were improper gut coiling, edema, as well as notochord and facial abnormalities. The 96-hr EC_{50} for malformations was 12–18 mg PFOS/L. Finally, tadpole growth was affected in the second and third assays, and the minimum concentrations inhibiting growth were determined to be 8.0 and 8.3 mg PFOS/L. The NOEC for growth was determined to be 5.2 mg PFOS/L.

Fish: Several acute toxicity studies with PFOS have been conducted using fish, including fathead minnows (*Pimephales promelas*), sheepshead minnows (*Cyprinodon variegatus*), bluegill sunfish (*Lepomis macrochirus*), and freshwater and marine rainbow trout (*Oncorhynchus mykiss*) (Table 2.5). Of the freshwater fish exposures, the fathead minnow was

the most sensitive species with a 96-hr LC_{50} of 9.1 mg PFOS/L, and an NOEC of 3.2 mg PFOS/L. After 96 hr of exposure, the sub-lethal effect of erratic swimming was noted in fathead minnows exposed to concentrations >5.6 mg PFOS/L (Drottar and Krueger 2000h).

Two acute toxicity tests with PFOS have been performed with rainbow trout in freshwater (Robertson 1986; Palmer et al. 2002a). Although the 96-hr LC_{50} values for PFOS in rainbow trout differed more than 2-fold between these two studies, the LC_{50} of 22 mg PFOS/L, as reported in the Palmer et al. (2002a) study, is more reliable than that reported in Robertson (1986), because the LC_{50} value in the Palmer et al. study (2002a) was calculated with measured PFOS concentrations rather than being based on nominal concentrations as were the values in the Robertson study (1986).

The sheepshead minnow, a brackish-marine species, has also been tested for PFOS acute toxicity, but was exposed to only one concentration of PFOS, 15 mg PFOS/L. This was the greatest concentration attainable in saltwater and required the addition of methanol (0.05%). No mortality was observed at this concentration after 96 hr of exposure, thus the 96-hr LC_{50} was reported as >15 mg PFOS/L, and the NOEC for sub-lethal effects was reported to be <15 mg PFOS/L (Palmer et. al 2002b). In another study, freshwater rainbow trout were acclimated over 5 d to a final salinity of 30% and were exposed to PFOS for 96 hr (Robertson 1986). For rainbow trout exposed to PFOS in saltwater, the 96-hr LC_{50} was calculated as 14 mg PFOS/L, and no sub-lethal effects were observed among rainbow trout at any PFOS concentration tested in this study. It should be noted that PFOS concentrations were not measured in this study and some of the nominal exposure concentrations were greater than the solubility of PFOS in saltwater.

2.3.2 Chronic Toxicity of PFOS to Aquatic Organisms

Microorganisms: The potential effects of PFOS on microorganisms in activated sludge have been determined by exposing microbes from a municipal wastewater treatment plant to various concentrations of PFOS (0.9 to 870 mg PFOS/L) (Schaefer and Flaggs 2000). After 3 hr of exposure, there was a 39% inhibition of the respiration rate, compared to controls, at the greatest concentration. However, the test concentration in this study exceeded the water solubility for PFOS, and as a result, based on known environmental concentrations, it is difficult to determine whether PFOS would cause any effects to microorganism communities (Table 2.6).

Microalgae: Many studies have been conducted to determine the toxicity of PFOS to aquatic microalgal species including phytoplankton and diatoms (Table 2.6). Since the lifecycle of most of these species is quite short (ranging from hr to d), these studies represent the measurement of chronic effects on multiple generations, even when the exposure period of these tests are short (72 to 96 hr). Toxicological endpoints that have been evaluated in these studies include growth (measured in terms of cell density or chlorophyll a content), and/or area under the growth curve over the test duration. Reported 96-hr EC_{50} values for freshwater microalgae (growth endpoint as measured by cell density) ranged from 48 to 263 mg PFOS/L. The 96-hr NOEC values for biomass ranged from 5.3 to 150 mg PFOS/L. Using biomass as the endpoint, the most sensitive species was *Selenastrum capricornutum* (NOEC=5.3 mg PFOS/L), whereas the diatom *Navicula pelliculosa* was the least sensitive species (NOEC=150 mg PFOS/L) (Boudreau et al. 2003a; Sutherland and Krueger 2001). When growth rate was evaluated as the test endpoint, 96-hr EC_{50} values ranged from 121 to 305 mg PFOS/L, and NOEC values ranged from 42 to 206 mg PFOS/L. Again, *S. capricornutum* was the most sensitive species, and *N. pelliculosa* was the least sensitive, using growth rate as the endpoint. Eeffects of PFOS on these

Table 2.6 Chronic toxicity of PFOS to aquatic organisms (95% confidence intervals in parentheses)

Trophic level	Test organism/ Species	Test Duration	Endpoint	NOEC (mg/L)	LOEC (mg/L)	$EC_{50}/LC_{50}/IC_{50}$ (mg/L)	Reference
Freshwater							
Microorganisms	Microorganism community	96 hr	Respiratory inhibition			>870	Schaefer and Flaggs 2000
Microalgae	*Selenastrum capricornutum*	96 hr	Growth (cell density)	42		68 (63-70)	Drottar and Krueger 2000a
		96 hr	Inhibition of growth rate	42		121 (110-133)	Drottar and Krueger 2000a
		96 hr	Growth (cell density)	5.3 (4.6-6.8)		48.2 (45.2-51.1)	Boudreau et al. 2003
		96 hr	Growth (chlorophyll a)	16.6 (8.5-28.1)		59.2 (50.9-67.4)	Boudreau et al. 2003
	Navicula pelliculosa	96 hr	Growth (cell density)	150		263 (217-299)	Sutherland and Krueger 2001
		96 hr	Inhibition of growth rate	206		305 (295-316)	Sutherland and Krueger 2001
	Chlorella vulgaris	96 hr	Growth (cell density)	8.2 (6.4-13.0)		81.6 (69.6-98.6)	Boudreau et al. 2003
	Zooplankton	35 d	Community	3.0			Boudreau et al. 2003b

	community		structure				
Macroalgae	*Myriophyllum spicatum*	42 d	Biomass, dwt	11.4		12.5 (6-18.9)	Hanson et al. 2005
		42 d	Root length, cm	11.4		16.7 (10.8-22.5)	Hanson et al. 2005
	Myriophyllum sibiricum	42 d	Biomass, dw	2.9		3.4 (1.6-5.3)	Hanson et al. 2005
		42 d	Root length, cm	0.3		2.4 (0.5-4.2)	Hanson et al. 2005
Invertebrate	*Daphnia magna*	21 d	Adult survival	5.3 (2.5- 9.2)		42.9 (31.7-56.4)	Boudreau et al. 2003a
	Chironomus tentans	10 d	Survival	0.05		>0.15	MacDonald et al. 2004
		10 d	Growth (chlorophyll a)	0.05		0.087	MacDonald et al. 2004
		20 d	Survival	0.0		0.092	MacDonald et al. 2004
		20 d	Growth (chlorophyll a)	0.0		0.094	MacDonald et al. 2004
Amphibians	*Rana pipiens*	16 wk	Partial life cycle	0.3	3	6.21 (5.12-7.52)	Ankley et al. 2004
Fish	*Pimephales promelas*	28 d	Microcosm	0.3	3.0	7.2 (5.2-9.2)	Oakes et al. 2005
		47 d	Early life stage	0.29	0.58		Drottar and Krueger 2000i

Marine

Microorganisms	*Anabaena flos-aquae*	96 hr	Growth (cell density)	93.8	131 (106-142)	Desjardins et al. 2001a
		96 hr	Inhibition of growth rate	93.8	176 (169-181)	Desjardins et al. 2001a
Microalgae	*Skeletonema costatum*	96 hr	Growth (cell density)	>3.2	>3.2	Desjardins et al. 2001b
Invertebrate	*Mysidopsis bahia*	35 d	Growth, # young produced	0.24		Drottar and Krueger 2000g

microalgal species were algistatic, since growth resumed when microalgae from the greatest PFOS treatments were placed in fresh growth media at the end of the exposure period. Furthermore, signs of aggregation or adherence of the cells to the flask were not observed, nor were there any noticeable changes in cell morphology at the end of the studies for any concentration evaluated.

Although concentration-response relationships for growth have been developed for freshwater algae, the marine diatom, *Skeletonema costatum*, was not affected by exposure to PFOS. In this study, a 96-hr EC_{50} could not be determined because at the greatest dissolved concentration attained under test conditions (3.2 mg PFOS/L), growth was not significantly inhibited. As a result, an analysis of the sensitivity between freshwater and marine algae could not be conducted.

In addition to evaluating PFOS toxicity in individual species of microalgae, the effects of PFOS have also been evaluated at the community level. In a controlled freshwater microcosm study, 0, 0.3, 3.0, 10, or 30 mg PFOS/L were administered to a zooplankton community for a total of 35 d. Results indicated that the zooplankton community structure was significantly altered by exposure to 10 or 30 mg PFOS/L. By day 35, the total number of zooplankton species decreased by an average of 45.1 and 74.3%, in the 10 or 30 mg PFOS/L treatments, respectively. Thus, the NOEC based on changes in zooplankton community structure was determined to be 3.0 mg PFOS/L (Boudreau et al. 2003b).

Aquatic Macrophytes: The chronic toxicity of PFOS was evaluated using two aquatic macrophytes, *Myriophyllum sibiricum* and *M. spicatum,* in a microcosm study (Hanson et al. 2005). Both species were exposed to PFOS concentrations ranging from 0.03 to 30 mg PFOS/L for 42 d; measured test endpoints were plant length, root number and length, node number, and

biomass, expressed as dry weight (dwt). Toxicity was observed at PFOS concentrations of >3 mg PFOS/L for *M. spicatum*, with the 42-d EC_{50} exceeding 12 mg PFOS/L. The NOEC was found to be consistently >11 mg PFOS/L. Toxicity for *M. sibiricum* was observed at PFOS concentrations of >0.1 mg PFOS/L, and the 42-d EC_{50} value was greater than 1.6 mg PFOS/L. The NOEC values of 2.9 and 0.3 mg PFOS/L were based on biomass and root length, respectively. In general, *M. sibiricum* was more sensitive than *M. spicatum*, regardless of the test endpoint.

Invertebrates: Life-cycle tests with *D. magna* have been conducted to evaluate the chronic toxicity of PFOS to freshwater aquatic invertebrates (Table 2.6). In one study, the 21-d LC_{50} was determined to be 43 mg PFOS/L, and the NOEC, based on adult survival, was estimated to be 5.3 mg PFOS/L (Boudreau et al. 2003a). In a separate life-cycle toxicity test of *D. magna*, the LC_{50} and NOEC, based on adult survival, were reported as 12-13 mg PFOS/L (Drottar and Krueger 2000f). In another life-cycle toxicity test with the saltwater mysid, the 35-d NOEC, based on growth and number of young produced, was 0.24 mg PFOS/L (Drottar and Krueger 2000g). In the course of life-cycle tests with both *D. magna* and the saltwater mysid, the young produced were briefly exposed to the same concentrations to which the respective first-generation adults were exposed. Survival was monitored for 48 hr (*D. magna*) or 96 hr (*M. bahia*). After 48 hr of exposure, results of a daphnid 2nd generation acute exposure produced an NOEC of 12 mg PFOS/L. The 2nd generation mysid shrimp were exposed to negative control, 0.055, 0.12, 0.24, or 0.53 mg PFOS/L for 96 hr. Survival was >95% for all 2nd generation mysids exposed to these test concentrations. The mysid 2nd generation acute exposure NOEC was 0.53 mg PFOS/L. These results indicated that the saltwater mysid may be more sensitive to PFOS than is freshwater *D. magna*. However, additional studies would need to be conducted to

better evaluate the toxicity of PFOS to 2nd generation organisms. Specifically, a greater range of PFOS concentrations is needed to further define the NOAEC (no observable adverse effect concentration) for 2nd generation mysid shrimp.

Amphibians: The survival and development of northern leopard frogs (*Rana pipiens*), from early embryogenesis through complete metamorphosis, has been investigated in a water exposure study with PFOS (Ankley et al. 2004). In tadpoles exposed to 0.03, 0.1, 0.3, 1.0, 3.0, or 10 mg PFOS/L, mortality was observed within 2 week of study initiation in the 10 mg PFOS/L treatment; >90% mortality was observed by week 4. Tadpole survival was not affected in any other treatment group. The mean LC_{50} at week 5 was 6.2 mg PFOS/L (5.1–7.5 mg PFOS/L). No statistically significant effects were observed for tadpoles exposed to <1.0 mg PFOS/L. However, there was a slight increase in time to metamorphosis and a decrease in total length of tadpoles at levels >3.0 mg PFOS/L. In addition, there was a slight increase in the incidence of thyroid follicle cell atrophy that was subtle and difficult to quantify. The PFOS-related chronic effects in leopard frogs occurred within a concentration range that has been shown to cause effects in fish and invertebrates.

Fish: Chronic toxicity data, from an early-life stage toxicity text, are available for fathead minnows (*Pimephales promelas*; Drottar and Krueger 2000i. In this study, eggs and larvae were exposed to PFOS in a flow-through system for 47 d. Measured water concentrations of PFOS in the various treatments were: <LOQ (limit of quantification), 0.15, 0.30, 0.60, 1.2, 2.4, or 4.6 mg PFOS/L. Fish exposed to PFOS at concentrations <0.30 mg PFOS/L showed no significant reduction in time to hatch, hatching success, and survival or growth. The PFOS did not affect percent hatch or growth of fry at any of the concentrations tested. Survival was the most sensitive endpoint in this study. Compared to controls, percent survival was significantly

reduced among fathead minnows exposed to concentrations >0.60 mg PFOS/L. Thus, the NOEC and LOEC (lowest observable effect concentration) for fathead minnows were determined to be 0.30 and 0.60 mg PFOS/L, respectively (Drottar and Krueger 2000i).

PFOS and other PFCs are globally ubiquitous and have been shown to be toxic to wide range of species, yet many unanswered questions still remain. The research described herein will answer some of these questions with respect to: the improvement of current analytical methods, the detection and associated risk of PFCs in South Korea and whether model PFCS such as PFOS and PFOA are good predictors of effects caused by exposure to other PFCs.

3 STANDARD PURITY AND RELATIVE RESPONSE FACTOR ANALYSIS OF PERFLUORINATED CHEMICALS

3.1 Introduction

Perfluorinated compounds (PFCs) have been produced in large quantities since the 1950s for a wide range of applications ranging from carpet coatings to fire retardants (Paul et al. 2009;Prevedouros et al. 2006). They have been found to be ubiquitous in both remote and urban environments (Giesy and Kannan 2001). They have been measured in various matrices including; human blood, plasma, serum, sediments, water, and wildlife tissue (Giesy and Kannan 2001;Higgins et al. 2005;Kannan et al. 2004). The most widely distributed, and also the most studied PFC is PFOS. Production of PFOS-based products was voluntarily halted by North America's largest producer; the 3M company in 2000 (3M 2000).

Although PFCs had been produced in large scale for more than 40 years, it was not until the late 1990's that researchers started detecting PFCs in the environment (Giesy and Kannan 2001). This was due to a number of factors including: lack of accurate and sensitive methods for extraction, lack of standards, especially isotopically-labeled ones, and lack of instrumentation with sufficient sensitivity (Martin et al. 2004a). With the advent of high performance liquid chromatography (HPLC) coupled to an electrospray-ionization tandem mass spectrometry (ESI-MS/MS), scientists could begin to accurately and routinely measure PFCs in the environment (Hansen et al. 2001).

Although in recent years there have been many advances in the quantification of PFCs, many issues still remain, such as matrix and isomer effects and impure standards (Arsenault et al. 2008a;Jacoby 2008). Production of PFOS-based products by electrochemical fluorination (ECF) that was commonly used by the 3M Company, results in a complex mixture of PFCs with a given purity of approximately 86 % (Seacat et al. 2003). This ECF-produced PFOS is comprised of 11

different isomers where approximately 70 % is the linear isomer and approximately 30 % contains different branched isomers (Benskin et al. 2007;Liu et al. 2007). These issues become increasingly important as scientists strive to measure PFCs in the environment at lower concentrations (Yamashita et al. 2004), which can often cause significant analytical bias.

This study was conducted to address three questions relevant to quantification of PFCs. The first objective was to determine concentrations of other PFCs present in specific lots of standard solutions of PFOS and perfluorooctanoic acid (PFOA). The second objective was to determine the relative response factors of isotopically-labeled analytes relative to their non-labeled counterparts. Lastly, the feasibility of using linear PFOS and PFOA standards to quantify ECF-produced, branched PFOS and PFOA was investigated. Individual 10 ng mL^{-1} solutions of PFOS and PFOA were diluted from original stock standards supplied by the 3M Company and were analyzed using HPLC-MS/MS under negative ion electrospray to detect any impurities present at proportions down to 0.1 %, relative to the major components. Standard solutions of isotopically and non-labeled materials were analyzed to compare response factors of isotopically-labeled analytes versus their non-labeled counterparts in three different matrices at equivalent concentrations: organic solvent (methanol), serum extract, and water. Standards were also reanalyzed, with an isotopically-labeled analyte concurrently present in the solvent, serum extract, and water with its non-labeled counterpart at the same concentration. This allowed for determination of suppression or enhancement of response due to possible interactions. Lastly, standard solutions of ECF-produced PFOS and PFOA were quantified using calibration curves prepared from standards consisting of linear only PFOS and PFOA.

3.2 Experimental

3.21. Chemicals and Standards

Omni-Solv grade methanol was purchased from EMD Chemicals (Gibbstown, NJ, USA). HPLC grade ammonium acetate was purchased from J.T. Baker (Phillipsburg, NJ, USA). The 3M Company provided ECF-produced PFOS (potassium salt, 86.9 %, lot # 217) and PFOA (ammonium salt, 95.2 %, lot # 332). Other PFC standards were obtained as follows: L-PFOA (Linear, free acid, 99.51 %, Oakwood Products), L-PFOA (free acid, >98 %, Wellington Laboratories), L-PFOSNa (sodium salt, >98 %, Wellington Laboratories), L-PFOSK (potassium salt, >98 %, Wellington Laboratories), PFOA[1,2,3,4 ^{13}C] (>98 %, Wellington Laboratories), PFOS[$^{18}O_2$] (RTI International), PFOS[1,2,3,4 ^{13}C], (>98 %, Wellington Laboratories), perfluorobutyric acid (PFBA) (99.2 %, Sigma-Aldrich), perfluoropentanoic acid (PFPA) (99.2 % Alfa Aesar), perfluorohexanoic acid (PFHxA) (97.7 %, Oakwood Products), perfluoroheptanoic acid (PFHpA) (98.2 %, Sigma-Aldrich), perfluorononanoic acid (PFNA) (98% Oakwood Laboratories), perfluorodecanoic acid (PFDA) (98%, Oakwood Laboratories), perfluoroundecanoic acid (PFUnA) (96.4 %, Oakwood Laboratories), perfluorododecanoic acid (PFDoA) (99.65 %, Oakwood Laboratories), perfluorobutane sulfonate (PFBS) (97.3 %, 3M Lot #2), perfluorohexane sulfonate (PFHxS) (98.6 %, 3M NB 120067-69), and linear perfluorodecane sulfonate (PFDS) (98 %, Wellington Laboratories). All standards were purchased by the 3M Company which then supplied them to the University of Saskatchewan for instrumental analyses.

3.2.2 HPLC-MS/MS Conditions

Analytical methods were optimized to allow simultaneous detection of all target analytes during a single run. Isomer separation was accomplished by use of an Agilent 1200 HPLC fitted with a Thermo Scientific Betasil C18 (100 x 2.1 mm, 5 um particle size) analytical column operated at 35 ^{o}C. Gradient conditions were used at 300 µL min^{-1} flow rate, starting with 60 % A

(2 mM ammonium acetate) and 40 % B (100 % methanol). Initial conditions were held for 2 minutes and then ramped to 20 % A at 18 min, held until 20 minutes, decreased to 0 % A at 21 min, increased to 100 % A at 22 min, held until 22.5 min, returned to initial condition at 23 min, and finally held constant until 26 min.

Mass spectra were collected using an AB SCIEX 3000 (Foster City, CA) tandem mass spectrometer, fitted with an electrospray ionization source, operated in the negative ionization mode. Chromatograms were recorded using multiple reaction monitoring (MRM) mode, where at least two transitions per-analyte were monitored. The following instrument parameters were used: desolvation temperature (450 ^{o}C), desolvation (curtain) gas 6.0 arbitrary units (AU); nebulizer gas flow 5 AU; ion spray voltage – 3500 V; collision gas 12 AU; and a dwell time of 40 msec. The optimal settings for collision energies and declustering potential were determined for each analyte's transitions to enhance detection and improve the signal to noise ratio. Quantification using these transitions was performed using Analyst 1.4.1 software provided by Sciex (Applied Bioscience, Foster City, CA).

3.2.3 Quality Control

To reduce instrument background contamination coming from the HPLC or solvents, a ZORBEX (Thermo Scientific, 50 x 2.1mm, 5 um particle size) column was inserted directly before the injection-valve, as adapted from Benskin et al. (2007). Blanks were run every 4 to 5 samples to check for carryover and background contamination, and at all times during analysis the blanks were found to be below the limit of quantification. Teflon coated lab-ware was avoided during all steps of standard solution preparation to minimize contamination of the samples. Samples were stored and analyzed in polypropylene auto-sampler vials fitted with

polypropylene septa (Canadian Life Science, Peterbrough, ON, CAN), as it has been shown that glass vials and polytetrafluoroethylene (PTFE) septa may cause loss of analyte and increased contamination, respectively (Yamashita et al. 2004).

3.2.4 Data and Statistical Analysis

Differences in PFOS and PFOA response factors were investigated using a one-way analysis of variance (ANOVA). If differences were found a Tukey post hoc test was also performed to determine were the differences existed. All statistical analyses were performed using the statistical software SYSTAT® 17.0 Package (SYSTAT Software Inc., Richmond, CA).

3.2.5 Purity Verification of Standards

Individual 100 ng mL^{-1} solutions of each of the PFOS and PFOA standards were prepared in a 50 % methanol, 50 % 2 mM aqueous ammonium acetate solution and were analyzed using HPLC-MS/MS under negative-ion electrospray to detect any impurities present down to 0.1 %, relative to the major components. PFOS and PFOA materials that were analyzed for purity verification are listed in Table 3.1. The target impurities which were monitored in each of the PFOS and PFOA standard solutions are given in Table 3.2. Calibration curves made from individual standard reference materials were used to quantify each of the target impurities.

Table 3.1 PFOA and PFOS Standards Evaluated for Target Impurities.

Name	*Synonym or Acronym*	*Formula*	*Vendor Source*	*Reported Purity*
Perfluorooctanoic acid (ammonium salt)	PFOA (C8 Acid)	$C_7F_{15}COO^-NH_4^+$	3M Lot #332	95.20%
Perfluorooctanoic acid (free acid)*	PFOA (C8 Acid)	$C_7F_{15}COOH$	Oakwood Products	99.51%
Perfluorooctanoic acid (free acid)*	PFOA (C8 Acid)	$C_7F_{15}COOH$	Wellington	99.51%
Perfluorooctane sulfonate (potassium salt)	PFOS	$C_8F_{17}SO_3^-K^+$	3M Lot #217	86.90%
L-Perfluorooctane sulfonate (sodium salt)*	PFOS	$C_8F_{17}SO_3^-Na^+$	Wellington	>98%
Perfluorooctane sulfonate (potassium salt)*	PFOS	$C_8F_{17}SO_3^-K^+$	Wellington	>98%
Isotopically labeled PFOA	PFOA [1,2 ^{13}C]	$C_6F_{13}[^{13}C]F_2[^{13}C]OOH$	Perkin Elmer	97.60%
	PFOA [1,2,3,4 ^{13}C]	$C_4F_9[^{13}C]F_2\ [^{13}C]F_2[^{13}C]F_2[^{13}C]OOH$	Wellington	>98%
Isotopically lableled perfluorooctane sulfonate	PFOS [$^{18}O_2$]	$C_8F_{17}S[^{18}O_2]O^-NH_4^+$	RTI International	>98%
	PFOS [1,2,3,4 ^{13}C]	$C_4F_9[^{13}C]F_2[^{13}C]F_2[^{13}C]F_2[^{13}C]F_2SO_3^-Na^+$	Wellington	>98%

***** Predominantly linear isomer

Table 3.2 Compounds Determined as Target Impurities.

Name	*Synonym or Acronym*	*Formula*	*Vendor Source*	*Given Purity*
Perfluorobutyric acid	PFBA (C4 Acid)	C_3F_7COOH	Sigma-Aldrich	99.20%
Perfluoropentanoic acid	PFPA (C5 Acid)	C_4F_9COOH	Alfa Aesar	99.20%
Perfluorohexanoic acid	PFHxA (C6 Acid)	$C_5F_{11}COOH$	Oakwood Products	97.70%
Perfluoroheptanoic acid	PFHpA (C7 Acid)	$C_6F_{13}COOH$	Sigma-Aldrich	98.20%
Perfluorooctanoic acid (ammonium salt)	PFOA (C8 Acid)	$C_7F_{15}COO^-NH_4^+$	3M Lot 217	95.20%
Perfluorononanoic acid	PFNA (C9 Acid)	$C_8F_{17}COOH$	Oakwood Products	98.02%
Perfluorodecanoic acid	PFDA (C10 Acid)	$C_9F_{19}COOH$	Oakwood Products	98.01%
Perfluoroundecanoic acid	PFUnA (C11 Acid)	$C_{10}F_{21}COOH$	Oakwood Products	96.40%
Perfluorododecanoic acid	PFDoA (C12 Acid)	$C_{11}F_{23}COOH$	Oakwood Products	99.65%
Perfluorobutane sulfonate (potassium salt)	PFBS	$C_4F_9SO_3^-K^+$	3M Lot#2	97.30%
Perfluorohexane sulfonate (potassium salt)	PFHxS	$C_6F_{13}SO_3^-K^+$	3M NB 120067-69	98.60%
Perfluorooctane sulfonate (potassium salt)	PFOS	$C_8F_{17}SO_3^-K^+$	3M Lot#217	86.90%
L-Perfluorodecane sulfonate; (predominantly linear)	PFDS	$C_{10}F_{21}SO_3^-Na^+$	Wellington	>98.00%

3.2.6 Unlabeled vs. Isotopically-Labeled Materials

Response Factor Verification: It has been reported that one of the major uncertainties in measurement of PFCs are the problems of ion suppression or enhancement as a result of matrix or co-elution effects (Arsenault et al. 2008a;Martin et al. 2004a). For this reason, an experiment was designed to measure the response factors of isotopically-labeled and native PFOS and PFOA standards present individually at a concentration of 100 ng mL^{-1} in methanol, serum extract, and reagent grade water. The same set of experiments was repeated where the native standards were present concurrently with the isotopically-labeled counterparts to see if there were any synergistic or antagonistic effects.

Individual standard solutions of unlabeled and isotopically-labeled materials (Table 3.1) were prepared to compare the response factors of isotopically-labeled analytes versus their non-labeled counterpart in three different matrices at equivalent 100 ng mL^{-1} concentrations: organic solvent (methanol), bovine serum extract, and 18 MOhm water. The solvent, serum, and water were extracted and checked for contamination prior to use, and all samples contained PFC concentrations below the limit of quantification. Response factors were calculated as shown below.

$$\text{Response Factor} = \frac{\text{Analyte Area Counts}}{\text{Analyte Amount}}$$

Response factor comparisons were done on equivalent mass transitions for the labeled and unlabeled PFCs, listed in Table 3.3.

Table 3.3 Mass Transitions of Isotopically Labeled and Unlabeled.

Isotopically Labeled Material	*Unlabeled Counterpart*	*Isotopically Labeled Transition(s)*	*Unlabeled Transition(s)*
PFOA [1,2 ^{13}C]	PFOA (C8 acid)	415>370	413>369; 413>219; 413>169
PFOA [1,2,3,4 ^{13}C]	PFOA (C8 acid)	417>372	413>369; 413>219; 413>169
PFOS [$^{18}O_2$]	PFOS (C8 sulfonate)	503>103, 503>84	499>99; 499>80; 499>130
PFOS [1,2,3,4 ^{13}C]	PFOS (C8 sulfonate)	503>99, 503>80	499>99; 499>80; 499>130

Synergistic Effects: After determining the response factor for the labeled materials present as individual analytes, the same set of experiments was repeated where the isotopically-labeled analyte was concurrently present in the solvent, serum extract, and water with its unlabeled counterpart at the same concentration, to determine if any synergistic effects were present.

3.2.7 Accuracy of Isomer Quantification

Standard solutions of 3M ECF PFOS (Lot# 217) and 3M ECF PFOA (Lot#332) were quantified using calibration curves prepared from standards consisting primarily of the linear isomer only (linear standards commercially obtained from Oakwood Products and Wellington Labs).

In this experiment, we used primarily linear (≥ 98 %) PFOS and PFOA standards to generate calibration curves, in order to quantify 3M ECF-produced multiple branched materials. When the linear standards used were present in salt form, great care was taken to insure that the concentration used were salt corrected, as many commercially available standards do not correct

for the salt form that is present. Known concentrations of branched PFOS and PFOA standards were analyzed as unknowns and quantified against their linear standard calibration curves. These results were then compared to their known concentrations.

3.3 Results and Discussion

3.3.1 Purity Verification of PFOS and PFOA Standards

The use of impure standards and the problems that this presents to the analysis for environmental samples has been well documented (Arsenault et al. 2008b;Martin et al. 2004a; Seacat et al. 2003). The manufacturers reported purities of PFOS and PFOA standards ranged from 86 % to over 98 %, but as Arsenault et al. (2008) reported these measurements are often based on an acid/base titration method after an ion exchange with a strong acid resin, which is not specific to PFOS or PFOA. Using these impure standards could not only result in improper measurement of the target analyte, but it could also result in significant contamination of an environmental sample with other PFCs that may be present such that they cannot be accurately quantified. The latter issue is becoming increasingly important as the sensitivity of newer more powerful instruments has allowed the detection of PFCs at lesser concentrations.

Impurities are in fact an issue with some PFC standards, in that the relative amount of each impurity can vary greatly depending on the source of the standard (Table 3.4). In general, our results matched the reported purity of standards, with a few notable exceptions. Our results also closely agree with a recent study that used calibration curves from standards of known purity to quantify PFOS standards of "unknown" purity (Arsenault et al. 2008). The largest difference between a reported and measured purity was for the isotopically-labeled PFOA produced by Perkin Elmer, which had relatively high levels of the C10 acid (Table 3.4).

Table 3.4 Purity Verification of PFOS and PFOA Materials (ng mL^{-1})

Compound	*PFBA*	*PFPA*	*PFHxA*	*PFHpA*	*Branched PFOA*	*PFNA*	*PFDA*	*PFUnA*	*PFDoA*	*PFBS*	*PFHxS*	*Branched PFOS*	*PFDS*
ECF PFOA (3M)	≤0.25	≤0.5	0.50	1.29	NA*	≤0.1	≤0.5	0.66	≤0.25	≤0.25	≤0.5	0.13	≤0.1
L-PFOA (Oakwood)	0.52	≤0.5	≤0.1	0.74	≤0.1	≤0.1	≤0.5	0.31	≤0.25	≤0.25	≤0.5	≤0.1	≤0.1
L-PFOA (Wellington)	≤0.25	≤0.5	≤0.1	≤0.1	≤0.1	≤0.1	≤0.5	0.22	≤0.25	≤0.25	≤0.5	≤0.1	≤0.1
ECF PFOS (3M)	1.21	0.82	1.28	0.59	0.59	0.11	≤0.5	0.24	≤0.25	1.72	7.31	NA	≤0.1
L-PFOS Na (Wellington)	≤0.25	≤0.5	≤0.1	≤0.1	≤0.1	≤0.1	≤0.5	0.32	≤0.25	≤0.25	≤0.5	≤0.1	≤0.1
L-PFOS K (Wellington)	≤0.25	≤0.5	≤0.1	≤0.1	≤0.1	0.14	≤0.5	0.29	≤0.25	≤0.25	≤0.5	≤0.1	≤0.1
PFOA [1,2 ^{13}C] (Perkin Elmer)	≤0.25	≤0.5	≤0.1	1.19	0.18	≤0.1	3.96	0.26	≤0.25	≤0.25	≤0.5	≤0.1	≤0.1
PFOA [1,2,3,4 ^{13}C] (Wellington)	≤0.25	≤0.5	≤0.1	≤0.1	0.21	≤0.1	≤0.5	0.33	≤0.25	≤0.25	≤0.5	0.85	≤0.1
PFOS [$^{18}O_2$] (RTI)	0.38	≤0.5	1.57	≤0.1	0.32	≤0.1	≤0.5	0.24	≤0.25	≤0.25	≤0.5	0.85	≤0.1
PFOS [1,2,3,4 ^{13}C] (Wellington)	1.83	0.94	0.89	≤0.1	≤0.1	≤0.1	≤0.5	0.26	≤0.25	≤0.25	≤0.5	0.10	≤0.1
Instrument Detection Limit (ng mL^{-1})	0.25	0.5	0.1	0.1	0.1	0.1	0.5	0.1	0.25	0.25	0.5	0.1	0.1

The biggest issue that could result from impurities being found in standards is to introduce multiple PFCs into an environmental sample that was spiked with an impure internal standard, such as for a lab matrix or field matrix spike sample. Based on the results of our study, under the worst case scenario, one could introduce approximately 14 ng (sum of impurities) of various PFC impurities from intentionally spiking 100 ng of 3M ECF-produced PFOS, where as the worst case scenario using supplier-grade standards could introduce approximately 7 ng (sum of impurities). This 14 ng and 7 ng, respectfully, could easily be enough to result in erroneous quantities of PFCs in environmental matrices, where often the concentrations reported are in the pg mL^{-1} range. For example, if a researcher spiked 100 ng of 3M ECF-produced PFOS into a 100 mL water sample, the concentration of the impurities unknowingly added could be 10- to 100-fold greater than those in the unaltered sample (Yamashita et al. 2004). This makes the choice of standards and sources of those standards very important, as using standards whose purity has only been verified using a non-specific acid/base titration technique could cause the overestimation of several different PFCs.

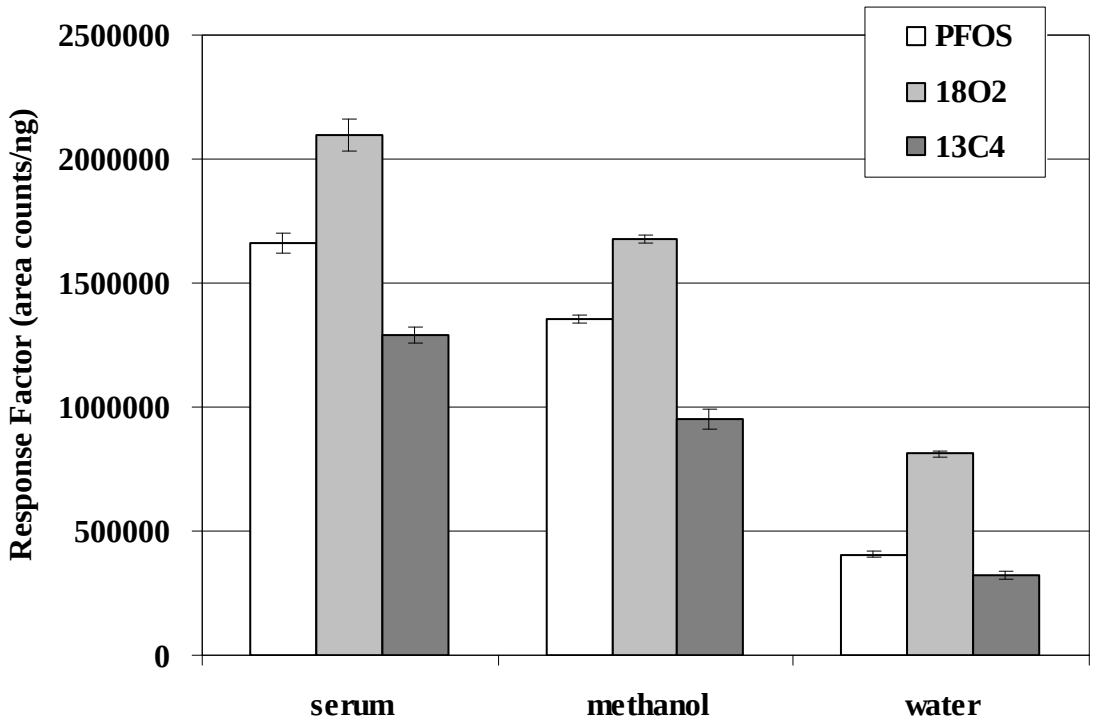
3.3.2 Response Factors of Labeled and Native PFOS and PFOA Standards

It should be noted that when discussing the issue of response factor comparisons for any analytes, determined by any chromatographic separation technique, that the subsequent results are only valid for that particular chromatographic separation method and detection technique. This arises from the issue of signal suppression or enhancement which can result from the presence of co-eluting, or closely eluting, interferents.

The individual response factors for PFOS and PFOA are presented in Figures 3.1 and 3.2. In general, all of the PFOS and PFOA standards (both labeled and non-labeled) followed the

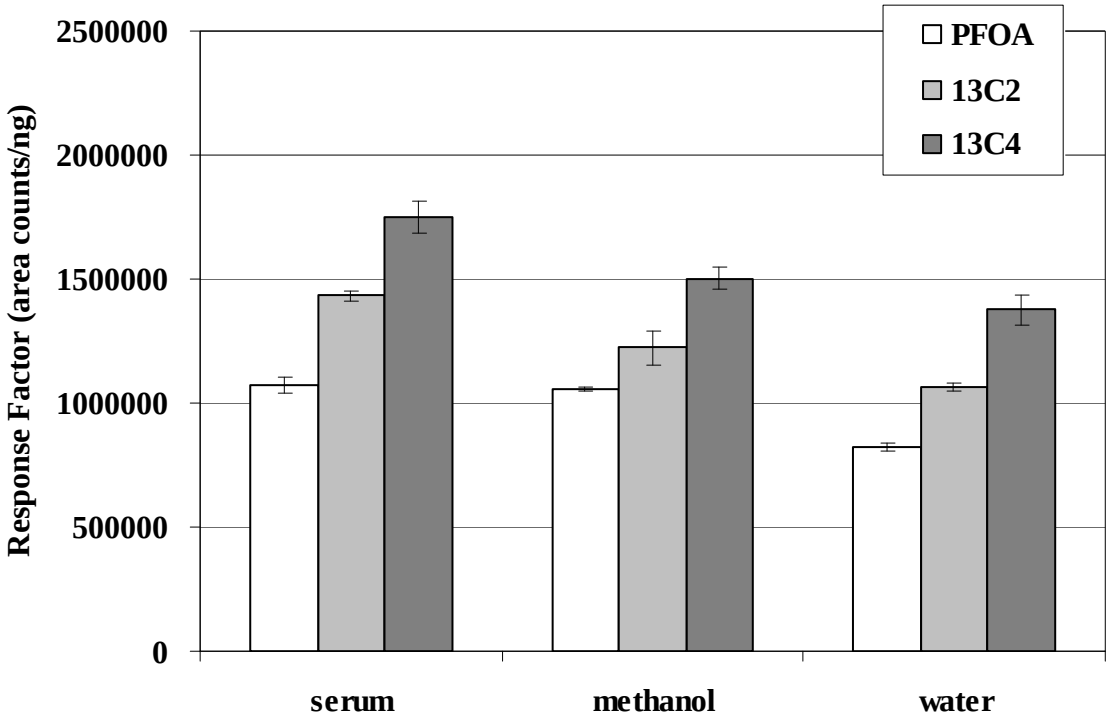
same pattern of relative response factors regardless of the matrix, although significant differences were seen when comparing standards within the same matrix and more importantly difference were seen when comparing the same standard present in different matrices. For the PFOS standards, response factors were the greatest in serum extract, then methanol, and were the lowest in water. For the PFOA standards, effects of each of the three matrices on response factors were less profound, although there were significant differences between PFOA in methanol and PFOA present in serum versus PFOA present in water. The most drastic differences seen between response factors as a result of matrix effects was the reduced response factor of native PFOS in water when compared to either methanol or serum. Native PFOA had a consistently lower response factor than either of its isotopically-labeled standards in all three matrices. The response factor of the $^{13}C_4$-labeled PFOA was also significantly higher than the $^{13}C_2$-labeled standard. For the PFOS standards, the $^{18}O_2$-labeled standard had a consistently greater response factor than either the $^{13}C_4$-labeled PFOS or the native standard in all three matrices. However, unlike the PFOA standards where both labeled analytes response factors were greater than the non-labeled standard, the $^{13}C_4$-PFOS standard was consistently lower than the native PFOS response factor. This suggests that there can be significant differences between the effects that isotopically labelling may have on the perfluorosulfonates and the perfluorocarboxylates. Although there were many significant differences as a result of matrix effects, further studies should be done using different instruments to confirm that these effects are in fact a result of matrix effects.

Figure 3.1 Individual Response Factors (Mean ± SD; n = 3) of PFOS Compounds.



The effects of combining labeled and non-labeled standards in methanol, serum, and water resulted in consistently greater response factors for both the $^{13}C_4$-labeled PFOA and the $^{18}O_2$-labeled PFOS than the other standards present as mixtures, regardless of the matrix (Figures 3.3 and 3.4). However, the relative response factors were similar when comparing both the individual experiments to the mixtures, so this increase in signal does not appear to be mixture-dependent. The mixed PFOA and PFOS standards followed similar trends for all of the analytes. However, all of the combined PFOS standards present in water were significantly suppressed, relative to the other matrices. The effect of having either $^{13}C_2$-PFOA or $^{13}C_4$-PFOA present with native PFOA appears to cause no significant effects on response factors, compared to the response of the native PFOA present individually.

Figure 3.2 Individual Response Factors (Mean ± SD; n = 3) of PFOA Compounds.



Results of these studies demonstrate that native PFOS and PFOA standards behave differently than their isotopically-labeled counter parts, and was especially true for $^{13}C_4$-PFOA and $^{18}O_2$-labeled PFOS standards, however it should be noted that additional experiments using multiple HPLC-MS/MS instruments, should be done to confirm that these differences are seen on all ESI-MS/MS instruments. This study also shows that, in general, there is little difference in the response factors of both labeled and non-labeled standards when they are present concurrently or independently. For both PFOS and PFOA the type of matrix the standards were prepared in had the most profound effects when comparing serum to both methanol and water, but significant differences were also seen when comparing between methanol and water. These differences in response factor as a result of matrix effects would be especially problematic if a researcher was using a methanol based calibration curve to measure concentrations of PFOS

and/or PFOA present in water and serum as it would cause an overestimation in the response for the serum samples and a underestimation in response for the water samples relative to the methanol based curve. This difficultly in accurately reporting concentrations of PFCs in water samples has been reported before (Taniyasu et al. 2005), and this present study further supports the need for better extraction methods, and the use of matrix matched calibration curves.

Figure 3.3 Response Factors (Mean ± SD; n = 3) of PFOA Compounds Present Concurrently with Other PFOA Standards.

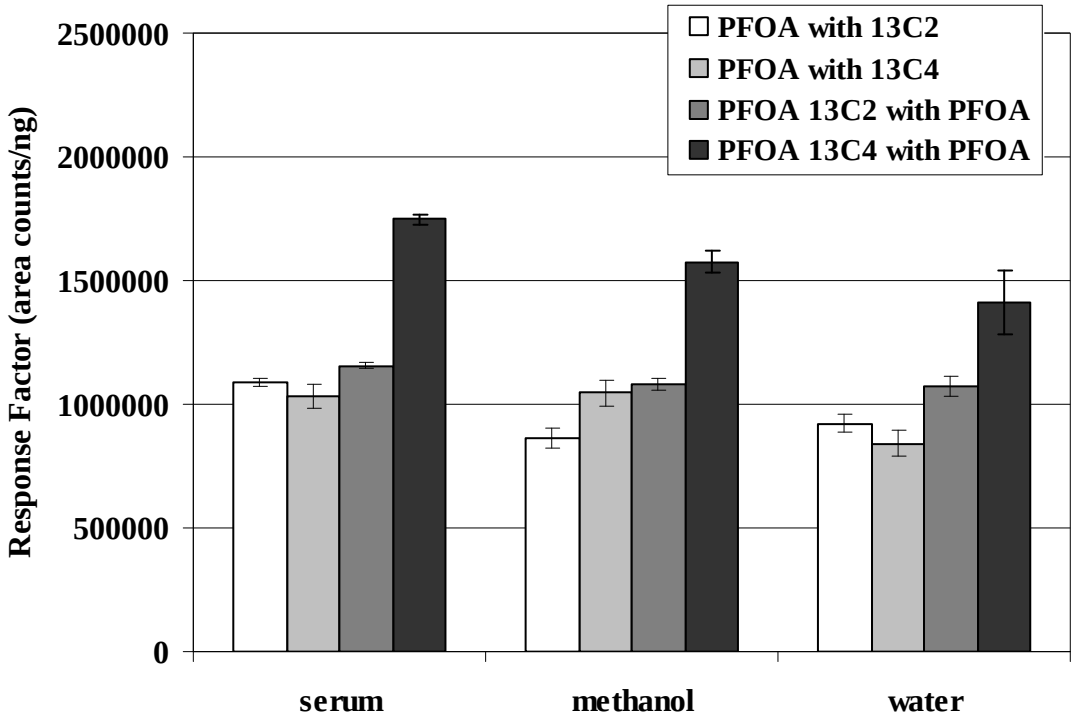
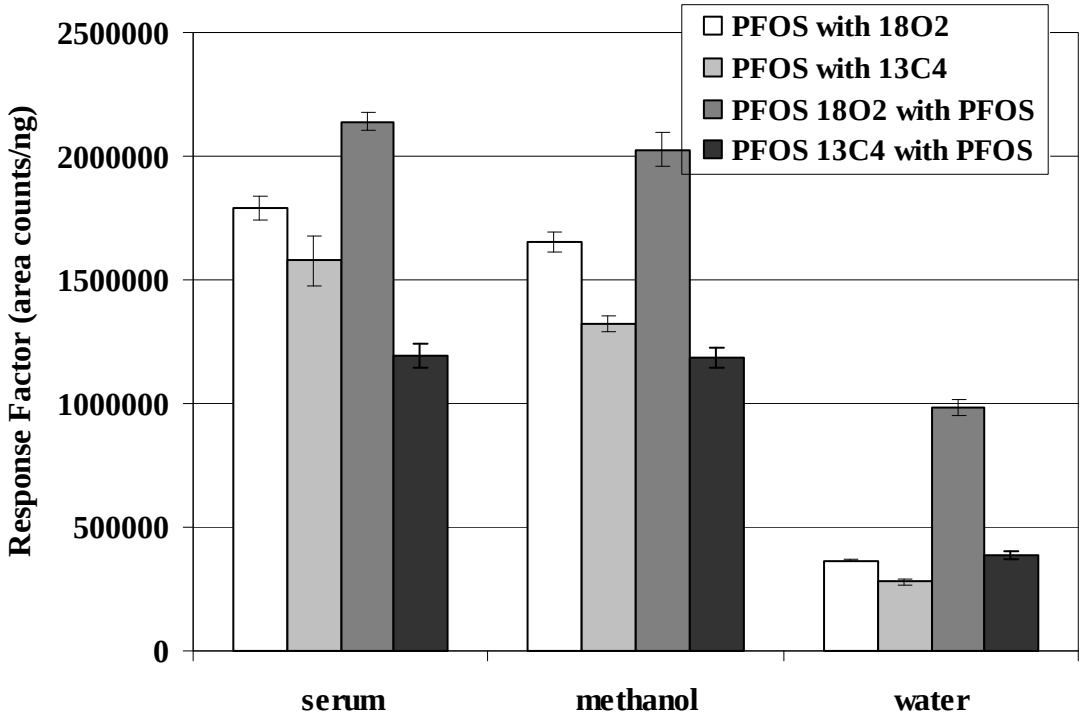


Figure 3.4 Response Factors (Mean ± SD; n = 3) of PFOS Compounds Present Concurrently with Other PFOS Standards.



3.3.3 Accuracy of Isomer Quantification against Linear PFOS and PFOA Standards

It is known that PFOS and PFOA exist in the environment as a mixture of multiple isomers (Benskin et al. 2007;De Silva and Mabury 2006;Furdui et al. 2008), and that these isomers may behave differently both analytically and toxicologically (Benskin et al. 2009b;O'Brien et al. 2011). However, most standards that are used to quantify environmental samples consist of a primarily linear standard. It has been assumed that these linear standards will behave similarly under HPLC-MS/MS conditions, and that using them to quantify multiple branched isomers will provide accurate measurements.

The use of linear PFOS or PFOA to quantify branched materials (sum of integration from all isomer peaks) resulted in an underestimation of approximately 21 % for PFOS (average of both linear standards) over a concentration range of 0.5 to 100 ng mL^{-1} and by 12 % for PFOA over a concentration range of 0.1 to 100 ng mL^{-1} (Figures. 3.5 and 3.6). Calibration curves made using L-PFOSNa resulted in concentrations that were closer to the actual known concentrations, than that which were reported using calibration curves generated using L-PFOSK, although the difference was relatively small; approximately 2 %, which is within the method uncertainty. The overall difference seen between the linear standards for both PFOS and PFOA, compared to that of the multiple branched compounds, is not likely due the counter ion present, as the 3M ECF PFOS material had the same counter ion present, as the linear Wellington standard. The implications of this under-prediction of multiple branched isomer measurement in environmental samples is significant and seems to be increasingly important at either end of the calibration curves, which adds uncertainty to the already more uncertain ends of the calibration curve, suggesting that there may be an optimal working range for these standards. This under-prediction alone may not be enough to cause gross misrepresentation of an analyzed environmental sample, but it is another one of the many issues that hamper accurate PFCs research.

Figure 3.5 Accuracy of Isomer Quantification Using Linear Only PFOA standards.

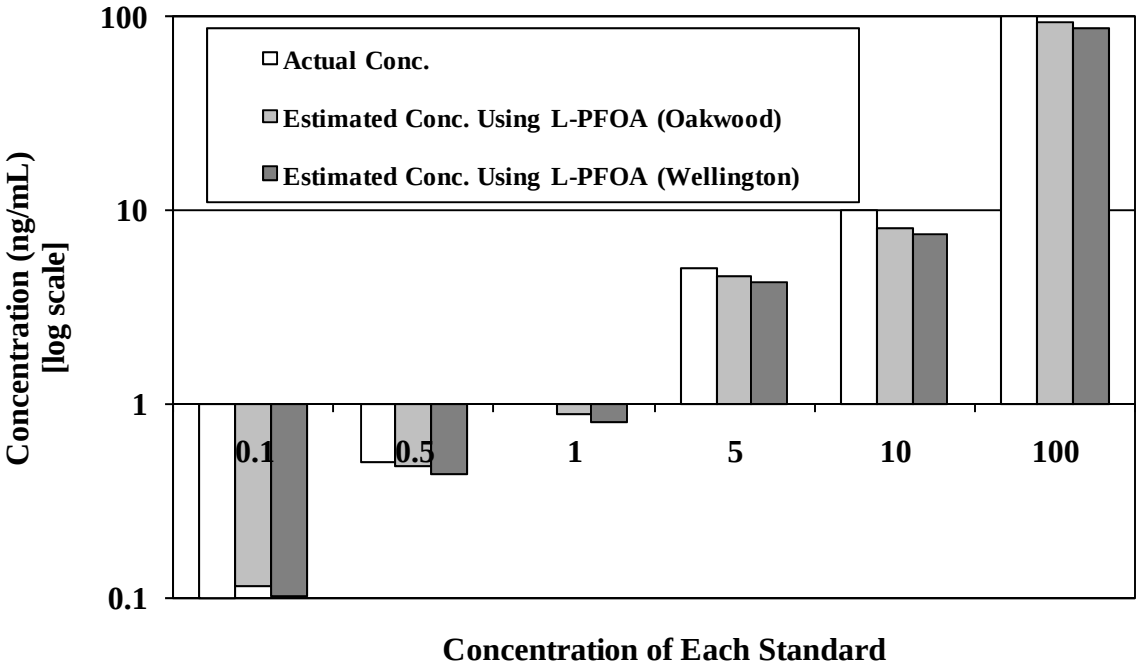
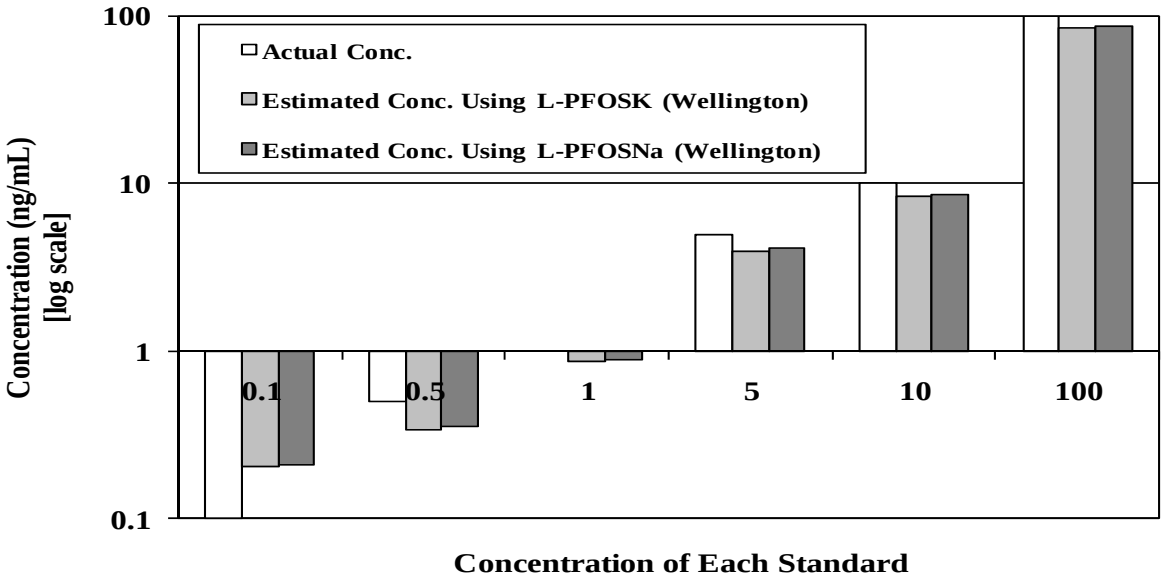


Figure 3.6 Accuracy of Isomer Quantification Using Linear Only PFOS Standards.



3.4 Conclusions

It was shown that depending on the sources, both PFOS and PFOA standards may contain significant amounts of impurities, which could potentially cause a significant over-estimation of many environmentally relevant PFCs. We have also shown that not all reported purities of standards are necessarily accurate and that the choice of suppliers is therefore, very important. The response factors of isotopically-labeled and native standards were compared, and it was observed that not all labeled analytes have the same response factor as their non-labeled complements, and that depending on the matrix, there may be significant suppression or enhancement of response, relative to a methanol based standard. Linear standards were used to quantify branched PFOS and PFOA standards which showed that in general, the use of linear standards may cause a marked under-prediction of concentrations, and that the working range of these standards may be limited. In general, these results show that unless great care is taken to ensure proper standard selection and use, researchers may generate data that could be significantly inaccurate.

4 PERFLUORINATED COMPOUNDS IN WATER, SEDIMENT, SOIL AND BIOTA FROM ESTUARINE AND COASTAL AREAS OF KOREA (2008)

4.1. Introduction

Perfluorinated compounds (PFCs) have been produced in relatively large quantities since the 1950s for a wide range of applications such as carpet coatings, food packaging, shampoos, paper, and fire-fighting foams (Giesy and Kannan 2001;Paul et al. 2009). PFCs make excellent surfactants due to their amphiphilic nature and resistance to breakdown. Some of these compounds are persistent in the environment, whereas others degrade to more environmentally stable compounds (Dinglasan et al. 2004). These properties arise from the characteristics imparted by the elemental fluorine atom, which is the most electronegative of the halogens (Giesy and Kannan 2002). These properties cause the C-F covalent bond, which makes up the back bone of any PFCs to be very strong and resistant to hydrolysis, photolysis, metabolism, and biodegradation (Kissa 2001). It is these properties that cause PFCs to be environmental persistent and hence have the potential to be bioacumulative (Kannan and Giesy 2002).

PFCs are globally ubiquitous in both remote and urban environments (Ellis et al. 2004;Giesy and Kannan 2001;Kannan and Giesy 2002;Yamashita et al. 2005). PFCs are present in various matrices including; human blood (whole, plasma and serum), sediments, water, and wildlife (Giesy and Kannan 2001;Kannan et al. 2004;Yamashita et al. 2005). Due to their widespread uses in many common products, PFCs are routinely found in the blood and serum of both occupationally and non-occupationally exposed people (Kannan et al. 2004;Karrman et al. 2005;Olsen et al. 2003a). The most widely distributed, and also the most studied PFC is perfluorooctanesulfonate (PFOS). While production of PFOS-based products was voluntarily

halted by North America's largest producer; the 3M company in 2000 (3M 2000), PFOS is still in environmental and human blood samples throughout Asia (Rostkowski et al. 2005;So et al. 2007;Yeung et al. 2006).

Although PFCs have been produced on a large scale for more than 40 yr, it was not until the late 1990's that researchers started detecting PFCs in the environment (Giesy and Kannan 2001). This was due to a number of factors including: lack of accurate and sensitive methods for extraction, lack of standards, especially isotopically labeled ones, and lack of instrumentation with sufficient sensitivity (Martin et al. 2004a). With the advent of high performance liquid chromatography (HPLC) coupled with electrospray-ionization tandem mass spectrometry, PFCs could be accurately and routinely measured in the environment (Hansen et al. 2001).

Previous studies have reported concentrations of PFCs in Korea to be relatively great (Kannan et al. 2004;Rostkowski et al. 2005;So et al. 2004;Yamashita et al. 2005;Yoo et al. 2009a). However, relatively little was known about sources, distribution and fate among matrixes including sediment, soil, water, and biota. As part of an ongoing study to determine the current status and extent of PFC concentrations, as well as potential for detrimental environmental effects in the Yellow Sea region of China and Korea, environmental samples were collected along the western coast of Korea during May of 2008. Locations were chosen based on previous work showing elevated concentrations of PFCs in the region and to detect possible point-source s (Im et al. 2004;Nakata et al. 2006;Rostkowski et al. 2005;So et al. 2004;Yoo et al. 2009a). Concentrations of PFOS and 12 other PFCs in environmental samples collected from estuarine and coastal areas of South Korea, were determined to assess the potential risk of PFCs to both humans and wildlife.

4.2. Materials and methods

4.2.1. Chemicals

Omni-Solv grade methanol was purchased from EMD Chemicals (Gibbstown, NJ, USA). HPLC grade ammonium acetate was purchased from J.T. Baker (Phillipsburg, NJ, USA). Sodium thiosulfate was purchased from EMD Chemicals (Gibbstown, NJ, USA). The internal standard consisted of PFOA [1,2,3,4 ^{13}C] (>98%, Wellington Laboratories), and PFOS [$^{18}O_2$] (RTI International). The external standard used for all matrix spikes was a mixture of 15 different PFCs (>98%, Wellington Laboratories) including perfluorobutyric acid (PFBA), perfluoropentanoic acid (PFPeA), perfluorohexanoic acid (PFHxA), perfluoroheptanoic acid (PFHpA), perfluorononanoic acid (PFOA), perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), perfluoroundecanoic acid (PFUnA), perfluorododecanoic acid (PFDoA), perfluorotridecanoic acid (PFTrDA), perfluorotetradecanoic acid (PFTeDA), perfluorobutane sulfonate (PFBS), perfluorohexane sulfonate (PFHxS), PFOS, and perfluorodecane sulfonate (PFDS).

4.2.2. Sample collection

Water, soil, sediment, and biota were collected from 8 estuarine and coastal areas along the Western side of South Korea during May of 2008 (Tables 4.1, 4.2 and Fig. 4.1). One liter of surface water was collected by dipping a clean, methanol rinsed polyporopylene (PP) 1 liter bottle just under the surface of the water. Residual chlorine in each water sample was reduced by adding 200 µl of 200 mg/ml of a sodium thiosulfate solution using a disposable PP syringe. Surface (top 1-5 cm) soil and sediment samples were collected using a clean methanol rinsed

stainless steel trowel. Samples were transferred and stored in clean PP bags. Biological samples were collected by hand in costal tidal pools and along the shore of inland water bodies, and were transferred and stored in clean PP bags. All samples were transported on ice at 4°C to the laboratory and frozen at -20 °C until analyses.

4.2.3. Extraction and cleanup

Water samples were extracted using Oasis HLB extraction cartridges (0.2 g, 6 cm^3) (Waters Corp., Milford, MA) as previously reported (So et al. 2004). In brief, the cartridges were pre-conditioned by eluting with 5 mL of methanol followed by 5 mL of nano-pure water at a rate of 2 drops a second. Five hundred mL of water was then spiked with 500 µL of 5 ng/mL of the internal standard and then loaded onto the cartridge, at a rate of 1 drop a second. The eluent was discarded. The cartridge was then washed with 5 mL of 40% methanol in water. The eluent was again discarded, and once complete was allowed to run dry. Lastly, the target fraction was eluted with 10 mL of methanol at a rate of 1 drop a second and collected in a 15 mL PP centrifuge tube. The resulting eluate was reduced to 1 mL under a gentle stream of nitrogen gas, and filtered using a disposable PP syringe, fitted with a disposable PP 0.2 µM filter (Millipore, Bedford, MA, USA). Samples were stored and analyzed in polypropylene auto-sampler vials fitted with polypropylene septa (Canadian Life Science, Peterbrough, ON, CAN), as it has been shown that glass vials and PTFE septa may cause loss of analyte and increased contamination, respectively (Yamashita et al. 2004).

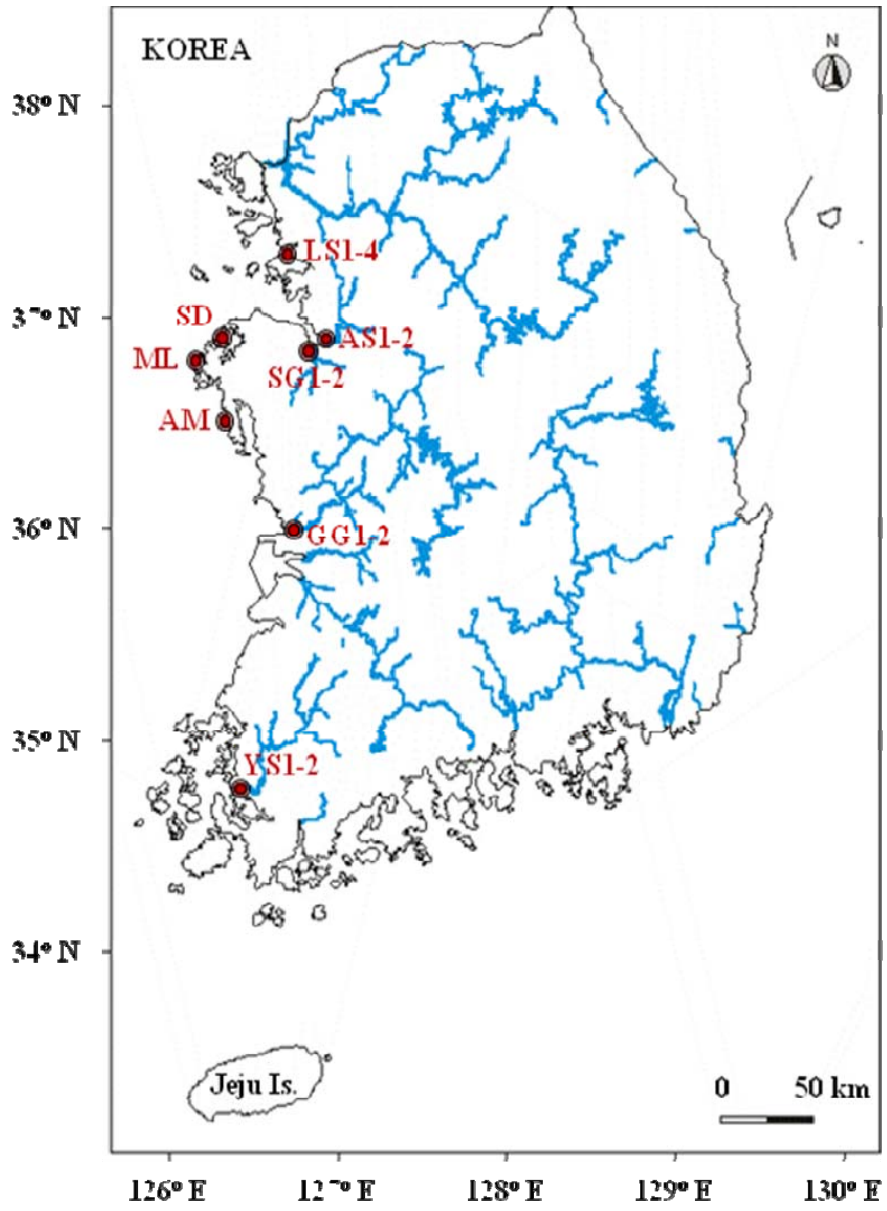
Table 4.1 Sampling details including location description and type of samples collected during the survey along the west coast of Korea.

Sampling				Samples			
Area	Date	Location	Geological Description	Water	Soil	Sediment	Biological (indv.)
Lake **S**hihwa	29-Apr	LS1	Outside of lake, Gyeonggi Bay	O	O	O	Surf Clam (7)
		LS2	Outside of lake, Gyeonggi Bay	O			Oyster (20)
		LS3	Inside of lake	O			Asian Periwinkle (50)
		LS4	Inside of lake	O			Asian Periwinkle (50)
Asan	30-Apr	AS1	Inside of lake	O		O	
		AS2	Outside of lake, Asan Bay	O	O	O	
Sap**G**yo	30-Apr	SG1	Inside of lake	O	O	O	
		SG2	Outside of lake, Asan Bay	O	O	O	Crab (10)
Sin**D**uri	30-Apr	SD	Beach	O	O	O	Striped Mullet (1)
Man**L**ipo	30-Apr	ML	Beach (Oil spill site)	O	O	O	Asian Periwinkle (200)
An**M**yundo	30-Apr	AM	Beach	O	O	O	Mussel (4)
							Blue Mussel (15)
							Neritid Gastropod (40)
							Asian Periwinkle (100)
Geum**G**ang	01-May	GG1	Upstream, inside of dam	O	O	O	
		GG2	Downstream, outside of dam	O	O	O	
Yeong**S**angang	01-May	YS1	Downstream, outside of dam	O	O	O	Asian Periwinkle (75)
							Rockfish (1)
		YS2	Upstream, inside of dam	O	O	O	
no. of location				15	11	12	9
no. of samples				15	11	12	21
							(573 individuals)

Table 4.2 Sampling details and detection results for samples collected during the survey along the west coast of Korea.

Area	Water		Soil		Sediment		Biological	
sampling location (n)	15		11		12		9	
samples analyzed (n)	15		11		12		21	
samples detected (n)								
PFBS	11	(73)	0	(0)	0	(0)	0	(0)
PFHxS	15	(100)	0	(0)	0	(0)	0	(0)
PFOS	15	(100)	4	(36)	3	(25)	21	(100)
PFDS	5	(33)	0	(0)	0	(0)	9	(43)
PFBA	6	(40)	0	(0)	0	(0)	0	(0)
PFPeA	4	(27)	0	(0)	0	(0)	0	(0)
PFHxA	14	(93)	1	(9)	0	(0)	17	(81)
PFHpA	13	(87)	3	(27)	2	(17)	4	(19)
PFOA	15	(100)	2	(18)	1	(8)	9	(43)
PFNA	15	(100)	1	(9)	0	(0)	1	(4.8)
PFDA	15	(100)	5	(45)	3	(25)	19	(90)
PFUnA	3	(20)	2	(18)	3	(25)	19	(90)
PFDoA	0	(0)	1	(9)	1	(8)	5	(24)
Total-Mean	10	(67)	1.5	(13)	1.0	(8)	8.0	(38)

Figure 4.1 Map of study area. Water, soil, sediment, and biota collected from 15 locations in 8 estuarine and coastal areas of Korea.



Soil and sediment samples were extracted using a previously published method (Higgins et al. 2005), with minor changes. Briefly, homogenized freeze-dried 1 g samples were transferred to 50 mL PP centrifuge tubes and spiked with 500 µL of a 5 ng/mL internal standard, to which 10 mL of a 1% acetic acid solution was added. Each vial was then vortexed, and placed

in a heated sonication bath for 15 min. After sonication, tubes were centrifuged at 3000 rpm for 2 min and the acetic acid solution was decanted into a new clean 50-mL PP tube. 2.5 mL of a 90:10 (v/v) methanol and 1% acetic acid mixture was then added to the original vial, and the vial was again vortexed mixed and sonicated for 15 min, before being centrifuged and decanted into the second tube. This process was repeated once more, and a final 10-mL acetic acid wash was preformed. All extracts were combined in the second tube before being passed through the solid phase extraction (SPE) cartridge in a similar fashion as was described above in the water extraction procedure.

Biological samples were extracted using an alkaline digestion SPE method (So et al. 2006). A 1 g aliquant of homogenized freeze-dried tissue was transferred to a 50-mL PP centrifuge tube and spiked with 500 µl of 5 ng/mL internal standard, and 30 mL of 0.01 N KOH/methanol was added to the tube. The mixture was then shaken at 250 rpm for 16 h. After this digestion 1 mL of the resulting tissue solution was added to a 1-L PP bottle containing 100 mL of nano-pure water and shaken thoroughly. This tissue-water mixture was then extracted using SPE cartridges as previously stated above.

4.2.4. Analysis

Analytical methods were optimized to allow simultaneous detection of all target analytes. Analyte separation was accomplished by use of an Agilent 1200 HPLC fitted with a Thermo Scientific Betasil C18 (100x2.1mm, 5 µm particle size) analytical column operated at 35 ^{o}C. Gradient conditions were used at 300 µL/min flow rate, starting with 60% A (2 mM ammonium acetate) and 40% B (100% methanol). Initial conditions were held for 2 min and then ramped to 20% A at 18 min, held until 20 min, decreased to 0% A at 21 min, increased to 100% A at 22

min, held until 22.5 min, returned to initial condition at 23 min, and finally held constant until 26 min.

Mass spectra were collected using an AB SCIEX 3000 (Foster City, CA) tandem mass spectrometer, fitted with an electro-spray ionization source, operated in the negative ionization mode. Chromatograms were recorded using MRM mode, and when possible at least two transitions per-analyte were monitored (Table 3.3). The following instrument parameters were used: desolvation temperature (450 ^{o}C), desolvation (curtain) gas 6.0 arbitrary units (AU); nebulizer gas flow 5 AU; ion spray voltage – 3500 V; collision gas 12 AU; and a dwell time of 40 msec. The optimal settings for collision energies and declustering potential were determined for each analyte's transitions. Quantification using these transitions was performed using Analyst 1.4.1 software provided by SCIEX (Applied Bioscience, Foster City, CA).

4.2.5. Quality control

To reduce instrument background contamination coming from the HPLC or solvents, a ZORBEX (Thermo Scientific, 50x2.1mm, 5 um particle size) column was inserted directly before the injection-valve, as adapted from Benskin et al. (Benskin et al. 2007). Blanks were run every 4 to 5 samples to check for carryover and background contamination. All blanks were found to be below the limit of quantification (LOQ). Teflon coated lab-ware was avoided during all steps of standard solution preparation to minimize contamination of the samples. The ions monitored, LOQ, limits of detection (LOD), and matrix spike recoveries for all of the chemicals of interest are given (Table 4.3).

Table 4.3 Target analytes of 13 perfluorinated compounds measured in the present study with QA/QC information including monitoring transitions, method detection limit, and matrix spike recovery for water, soil and sediment, and biological samples.

Analyte	Acronym	Monitoring Transitions	Method Dection Limit			Matrix Spike Recovery		
			Water (ng/L)	Soil/Sed (ng/ml)	Biological (ng/ml)	Water (%)	Soil/Sed (%)	Biological (%)
Perfluorobutanesulfonate	PFBS	299 → 99, 80	1	1	1	94	32	97
Perfluorohexanesulfonate	PFHxS	399 → 99, 80	0.2	0.5	1	137	134	113
Perfluorooctanesulfonate	PFOS	499 → 99, 80	0.2	0.5	0.1	101	95	89
Perfluorodecanesulfonate	PFDS	599 → 99, 80	0.2	0.5	0.1	101	43	70
Perfluorobutanoic acid	PFBA	213 → 169	2	1	1			
Perfluoropentanoic acid	PFPeA	263 → 219	2	1	1			
Perfluorohexanoic acid	PFHxA	313 → 269	1	0.1	1	85	78	73
Perfluoroheptanoic acid	PFHpA	363 → 319, 169	1	0.1	0.1	103	123	112
Perfluorooctanoic acid	PFOA	413 → 219, 169	1	0.5	0.5	88	89	88
Perfluorononanoic acid	PFNA	463 → 419, 219	2	1	1	133	135	133
Perfluorodecanoic acid	PFDA	513 → 469, 269	0.2	0.1	0.1	93	106	75
Perfluoroundecanoic acid	PFUnA	563 → 269, 219	2	0.5	1	112	53	93
Perfluorododecanoic acid	PFDoA	613 → 569, 319	2	0.1	0.5	77	70	47

4.3. Results and discussion

4.3.1. PFCs in water

Although 13 different compounds were investigated, the following discussion will focus primarily on PFOS and PFOA since these compounds were consistently found at the greatest concentrations. Occurrence and concentrations of PFCs in samples collected from western Korea during the summer of 2008 are summarized (Tables 4.2, 4.4-4.5). PFHxS, PFOS, PFOA, PFNA, and PFDA were detected in all water samples. Concentrations of PFOS and PFOA in water ranged from 5.2 to 450and from 3.3 to 50.1 ng/L, respectively. The mean concentrations of PFOS and PFOA were 59.5 and 20.6 ng/L, respectively. The next greatest mean PFC concentration was 10.0 ng PFHpA/L with a range of 1.11 to 47.2 ng/L.

The greatest concentration of PFOS (450 ng/L) was found at location AS1 which is located in the Asan reservoir adjacent to the city of Asan. This sample showed relatively great concentrations of all of the PFCs monitored, and had a total PFC (sum of all 13 compounds; ∑PFC) concentration of approximately 700 ng/L, which is among the greatest concentrations ever reported in water from this region (Table 4.6). Due to poor water circulation and a large amount of industrial development in the vicinity, Asan reservoir has had poor water quality since completion of dike construction in the early 1970s. Due to dilution, corresponding concentrations of PFCs at the outer location AS2 were less than those at more inner location. The least concentrations were found at SinDuri, which represents coastal ocean water where concentrations of PFOS and PFOA were 5.21 and 2.95 ng/L, respectively, with the concentration of ∑PFC less than 10 ng/L.

Table 4.4 Concentrations (ng/L) of PFCs detected in water samples collected from the west coast of Korea.

Location	PFBS	PFHxS	PFOS	PFDS	PFBA	PFPeA	PFHxA	PFHpA	PFOA	PFNA	PFDA	PFUnA
LS1	4.68	2.27	103				3.63	2.40	9.58	2.79	1.27	
LS2	1.35	1.18	8.69				1.66	1.11	3.30	1.83	0.31	
LS3	1.43	1.34	66.3				1.70	1.36	4.54	3.43	4.23	
LS4		3.66	53.7				3.79	3.58	15.1	5.58	5.22	2.88
AS1	39.8	41.8	450		3.98		47.0	29.7	50.1	14.3	15.4	2.84
AS2	8.00	8.58	36.2			6.23	8.89	6.02	14.7	3.82	0.83	
SG1		2.53	12.8	0.20	4.48		5.15	3.87	68.6	2.61	0.53	
SG2	7.84	8.18	28.5		3.10		13.0	10.6	35.1	5.49	1.04	
SD		0.58	5.21				1.28		2.95	1.38	0.23	
ML		0.38	4.11	0.23					3.04	2.03	0.26	
AM	2.05	1.76	6.32	0.22	3.90		4.98	3.89	10.6	2.65	0.34	
GG1	3.30	4.51	48.4	0.22		3.25	5.43	4.94	39.7	7.48	4.38	3.52
GG2	4.68	4.83	18.3	5.28		2.39	5.91	4.01	28.4	4.66	1.08	
YS1	1.51	1.52	9.83		9.55	6.09	4.86	47.2	6.09	4.37	0.67	
YS2	2.57	10.2	40.5		4.66		8.12	11.5	17.0	6.13	1.16	
min	< 1.0	0.38	4.11	< 0.2	< 2.0	< 2.0	< 1.0	< 1.0	2.95	1.38	0.23	< 2.0
max	39.8	41.8	450	5.28	9.55	6.23	47.0	47.2	68.6	14.3	15.4	3.52
mean	7.02	6.22	59.5	1.23	4.95	4.49	8.25	10.0	20.6	4.57	2.46	3.08

Table 4.5 Concentrations (ng/mL) of PFCs detected in biological samples collected from the west coast of Korea.

Location	Species	Samples	PFOS	PFDS	PFBA	PFHXA	PFHpA	PFOA	PFNA	PFDA	PFUnA	PFDoA
LS1	Surf Clam	soft tissue	4.50	0.14		1.52				0.80	2.24	
LS2	Oyster	soft tissue	1.53			5.04						
LS3	Asian Periwinkle	soft tissue	6.50	0.79		1.15		1.45		1.41	2.33	0.75
LS4	Asian Periwinkle	soft tissue	8.40			1.58		1.10	1.27	2.08	3.88	1.58
SG2	Crab	eggs	8.89			1.07		0.51		0.49	1.97	
		shells	1.14							0.32		
		soft tissue	1.30					0.76		1.46	1.81	
SD	Striped Mullet	fillet	8.83							0.13	1.28	
		intestines	266			34.6					1.46	
		liver	612			10.0				0.13	2.38	
ML	Asian Periwinkle	soft tissue	0.26			4.08					1.32	0.99
AM	Mussel	soft tissue	0.77	0.21		4.97	0.96	0.94		0.28	1.61	0.54
	Blue Mussel	soft tissue	0.34	0.22		3.81				0.67	1.09	
	Neritid Gastropod	soft tissue	0.75	0.15		3.75		0.62		1.31	1.31	
	Asian Periwinkle	soft tissue	0.59		5.81	5.01	0.98	0.69		1.02	4.40	
YS1	Rockfish	fillet	2.97	0.44		3.74	0.52	1.46		0.53	1.86	1.78
		intestines	11.2	0.18		2.45				1.27	2.27	
		liver	15.3				0.18			0.19	2.36	
		gills	99.2	0.24		9.43				0.21	1.40	
YS1	Asian Periwinkle	soft tissue	233			2.42				0.56	1.26	
min			0.26	<0.1	<1.0	<1.0	<0.1	<0.5	<1.0	<0.1	<1.0	<0.5
max			612	0.79	5.81	34.6	0.98	1.46	1.27	2.08	4.40	1.78
mean			64.2	0.30	5.81	5.91	0.66	0.94	1.27	0.76	2.01	1.13

Table 4.6 Concentrations (ng/mL) of PFOS and PFOA in water samples reported in Asian countries including Korea, China, and Japan

Sampling Location	Area Type	Year	n	PFOS Mean	PFOS Min	PFOS Max	PFOA Mean	PFOA Min	PFOA Max	References
Korea										
Lake Shiwa Area	Streams	2004	21	89.1	8.03	651	19.2	5.21	61.7	Rostkowski et al., 2006
	Lake	2004	5	12.9	7.33	18.3	6.14	1.67	10.9	Rostkowski et al., 2006
	Gyeonggi Bay	2004	5	5.21	8.26	2.24	0.47	0.44	0.50	Rostkowski et al., 2006
West Coast	Open ocean	2003	5	147	0.62	730	65.7	1.30	320	So et al., 2004
Southern Coast	Open ocean	2003	6	0.75	0.04	2.30	4.84	0.24	11.0	So et al., 2004
Korean Coast	Open ocean	2002-2004	10		0.04	2.53		0.24	11.4	Yamashita et al., 2005
Korean Coast	Lake, rivers, coastal	2008	15	59.5	4.11	450	20.6	2.95	68.6	This study
China										
Shanghai	Tap water	2006-2008	5	7.60			78.0			Mak et al., 2009
Nanjing	Tap water	2006-2008	5	0.94			5.90			Mak et al., 2009
Hong Kong	Tap water	2006-2008	5	7.00			1.10			Mak et al., 2009
Shenyang	Tap water	2006-2008	3	0.39			0.79			Mak et al., 2009
Beijing	Tap water	2006-2008	4	0.04			0.44			Mak et al., 2009
Hong Kong Coast	Open ocean	2002-2004	12		0.07	2.60		0.67	5.45	Yamashita et al., 2005
China Coast	Open ocean	2002-2004	14		0.02	9.68		0.24	15.3	Yamashita et al., 2005
South China Sea	Open ocean	2002-2004	2		0.01	0.11		0.16	0.42	Yamashita et al., 2005
Western Pacific	Open ocean	2002-2004	2		0.05	0.08		0.14	0.14	Yamashita et al., 2005
Dalian	Rain water	2006	2	61.5	9.92	113	36.9	32.9	40.8	Liu et al., 2009
Dalian	Snow	2006	3	120	42.2	138	12.6	9.16	16.7	Liu et al., 2009
Dalian	Snow	2007	2	72.8	108	37.5	32.2	7.74	56.7	Liu et al., 2009
Dalian	Coastal surface water	2006	14	0.23	0.10	0.96	0.56	0.27	2.12	Ju et al., 2008
Guangzhou	Pearl River	2005	12	23.1	0.90	99.0	4.28	0.85	13.0	So et al., 2007

Nanjing	Yangtze River	2005	6	0.36	0.33	0.39	2.25	2.00	2.60	So et al., 2007
Shanghai	Yangtze River	2005	6	5.14	0.62	14.0	5.14	0.62	14.0	So et al., 2007
Japan										
Osaka	Tap water	2006-2008	3	1.60			18.0			Mak et al., 2009
Tokyo	Tap water	2006-2008	1	1.60			40.0			Mak et al., 2009
Tokyo Bay	Open ocean	2002-2004	8		0.38	57.7		1.80	192	Yamashita et al., 2005
Offshore of Japan	Open ocean	2002-2004	4		0.04	0.07		0.14	1.10	Yamashita et al., 2005
Survey of Japan	River samples	2002	126	2.37	0.30	157				Saito et al., 2003
Survey of Japan	Costal sea water	2002	16	1.52	0.20	25.2				Saito et al., 2003
Tokyo Bay	Surface water	2002	4	26.0	8.00	59.0				Taniyasu et al., 2003
Osaska Bay	Surface water	2002	3	8.70	4.00	21.0				Taniyasu et al., 2003
Lake Biwa	Surface water	2002	3	3.80	4.00	7.40				Taniyasu et al., 2003
Ariake Bay	Surface water	2002	4	4.80	9.00	11.0				Taniyasu et al., 2003
Kyoto Area	River water	2005	5	6.50	7.90	110	58.6	5.12	10.0	Senthilkumar et al., 2007
Yodo River Basin	Surface water	2004-2005	81	3.90	0.40	123	4.20	2600	29.9	Lien et al., 2008
Survey of Japan	Sewage effluent	2005	5	179	42	635	46.4	10.0	68.0	Murakami et al., 2008

Concentrations of PFCs in Korean waters were relatively greater than those in other areas such as the North American Great Lakes, South China Sea, Arctic, Antarctic, and Pacific oceans (Roulanger et al. 2004;So et al. 2004;Yamashita et al. 2005). For example, the greatest concentration of PFOS ever reported in water from Tokyo Bay, Japan was 59 ng/L, while the mean PFOS concentration from all of the Korean locations was approximately 59.5 ng/L (Taniyasu et al. 2003). Concentrations measured in this study were similar to those reported previously for Korea (Rostkowski et al. 2005;Yoo et al. 2009a), accept for greater concentrations of PFOS in water Lake Shihwa. Lake Shihwa is a man-made lake on the outskirts of Seoul, which is heavily used and influenced by local industrial practices, and has been the focus of several previous studies (Rostkowski et al. 2005;Yoo et al. 2009a). Concentrations of PFCs measured in our study were similar to those previously measured in streams around Lake Shihwa and not what was measured in the actual lake and surrounding Gyeonggi Bay. In general, the ratio of PFOS to PFOA (mean of >4) agrees well with previously published values (Figs. 4.2 and 4.3), but concentrations of PFOS measured in waters of Lake Shihwa were 6-fold greater in 2009 than they had been in 2006 (Rostkowski et al. 2005).

Figure 4.2 Pattern of relative concentrations of individual PFCs in water collected from the west coast of Korea.

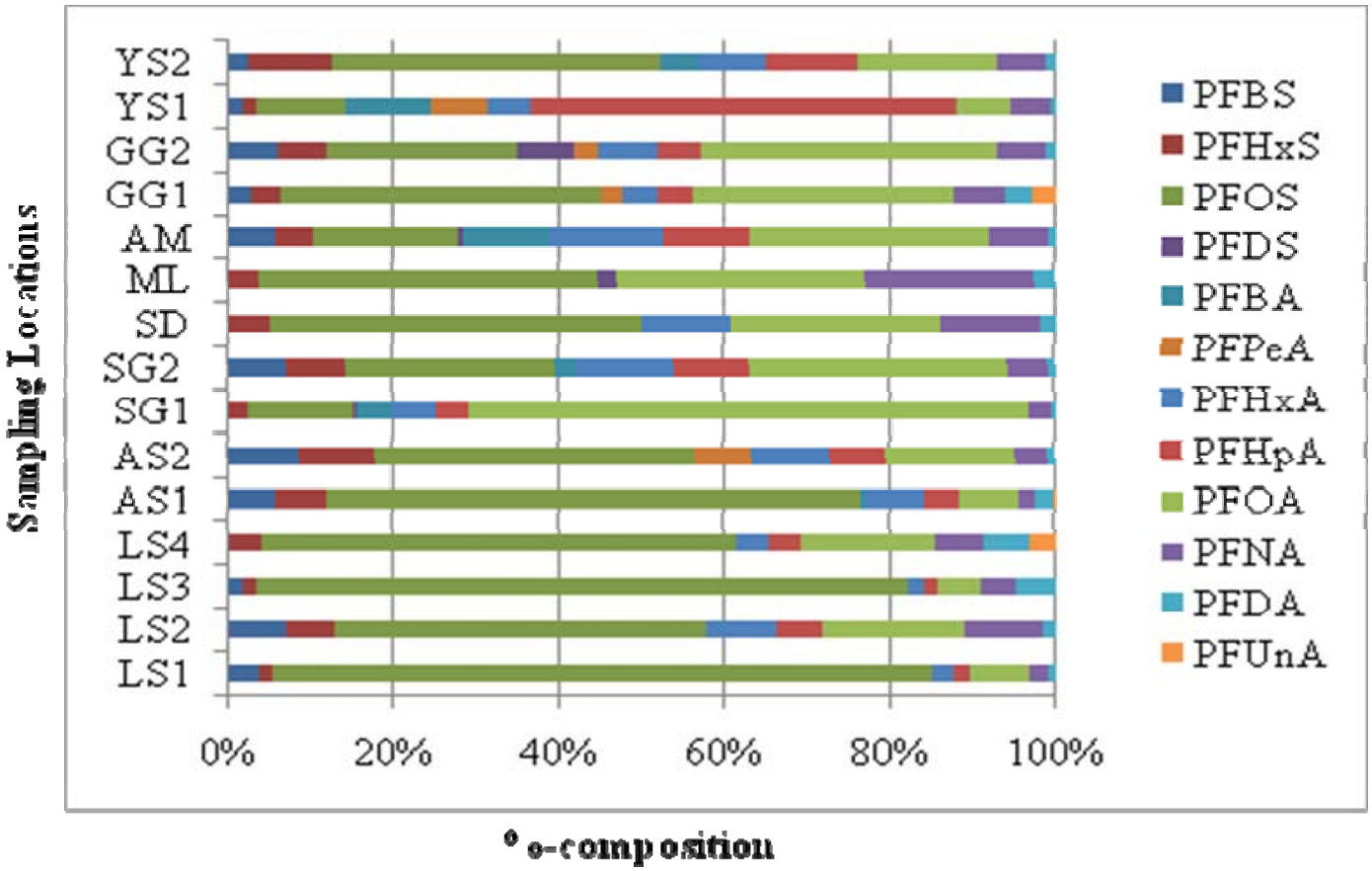
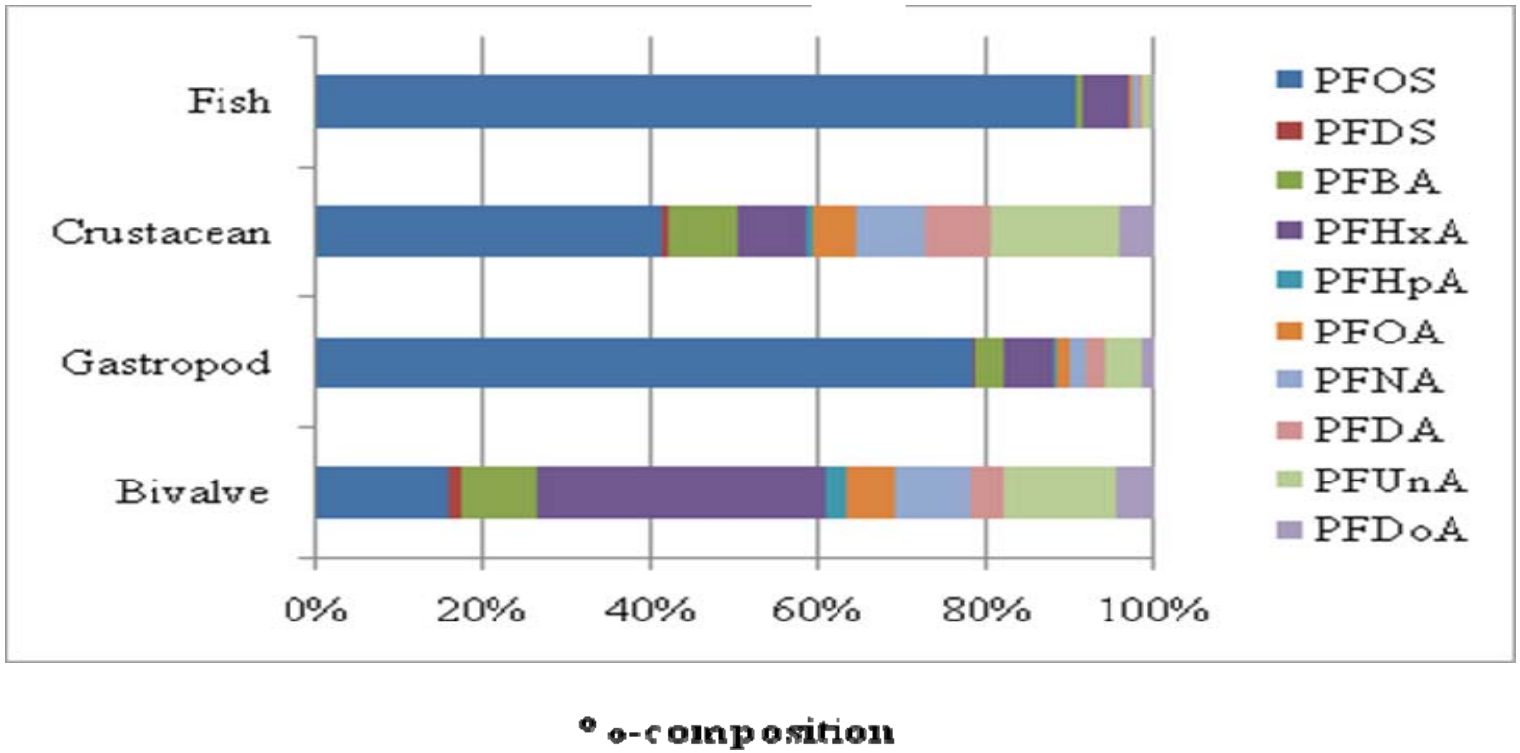


Figure 4.3 Pattern of relative concentrations of individual PFCs fishes, crustaceans, gastropods, and bivalves collected from the west coast of Korea.



4.3.2. PFCs in soils and sediments

Concentrations of PFCs in soils and sediments were generally less than the LOQ, but when detected were generally less those found in biota. Concentrations were comparable to those previously reported in other areas of Asia but slightly less than those reported in Europe and the United States (de Voogt and Van Roon 2005;Higgins et al. 2005;Nakata et al. 2006;Senthilkumar et al. 2005). When PFCs were detected, the heavier PFCs such as PFOS and PFDA were predominant, although only 5 soil and 3 sediment samples contained detectable concentrations of PFCs. There did not seem to be a difference between soils and sediments, neither had a PFOS concentration greater than 2.0 ng/g. In general, it appears that soil and sediment samples in Korea contain only small amounts of PFCs and do not appear to a contribute significantly to the exposure of benthic or terrestrial organisms.

4.3.3. PFCs in biota

Similar to sediments, concentrations of PFCs in biota were relatively small. PFOS was the predominant PFC in biota with a mean concentration of 64.2 ng/g dw and values ranging from 0.26 to 612 ng/g. Concentrations of PFOA in biota were less than those of PFOS with concentrations ranging from less than the MDL to 1.46 ng/g. Other PFCs consistently detected were PFHxA, PFDA and PFUnA which had maximum concentrations of 34.6, 2.08 and 4.40 ng/g, and average concentrations of 5.91, 0.76, and 2.01 ng/g, respectively. The greatest concentration of PFOS (612 ng/g) was found in the liver of fish collected from SinDuri Beach and the second greatest concentration (266 ng/g) was found in the intestines of fish collected from the same location, indicating point sources near SinDuri Beach. Also, soft tissues of Asian

Periwinkles (*Littorina brevicula*) and gills of Rockfish (*Sebastes schlegeli*) from the downstream of YeongSangang location (YS1) contained relatively great concentrations of PFOS (233 and 99.2 ng/g, respectively), which suggested accumulation of PFOS in filter-feeding biota across the water column. Concentrations of PFOS from all other locations were relatively low. In Lake Shihwa and YeongSangang, higher trophic level organisms contained the greater the concentrations of PFCs. For example, at YeongSangang the mean concentration of most PFCs was found to be greater in the Rockfish than those in the Asian Periwinkles (Table 4.5).

Concentrations of PFOS in fish collected from Korean waters during this study were similar to those observed previously in Korea and Japan (Nakata et al. 2006;Taniyasu et al. 2003;Yoo et al. 2009a). However, the concentration of 612 ng/g PFOS measured in this study is among the highest ever reported in fish from this region. In a recent study, concentrations of PFOS in fish from the east-central United States reported a median whole organism PFOS concentration of 37 ng/g (ww), which is 5.2-fold less than the mean concentration of all fish sampled in this study and 16.5-fold less than the maximum concentration in fish from SinDuri.

4.3.4. Pattern of relative concentrations

PFOS and PFOA were the most dominant PFCs observed. PFOS was the dominant PFC in both water and biota (Figs. 4.2 and 4.3). PFOS was the dominate PFC in 10 of the 15 water samples, whereas PFOA was dominate in water from only 5 locations. This observation is different than what is often observed at other locations, where PFOA is often the dominate PFC in water (Yamashita et al. 2005). This relatively large percentage of PFOS in water samples, suggests localized sources that are unique to this region.

Site-specific, apparent bioconcentration factors (BCF) were calculated for PFOS in fish by dividing the concentration of PFOS in fish by that in water at the same location. Due to a lack of fish samples BCFs could only be calculated for PFOS at two locations. The mean BCF for both locations was 33 000 with a maximum value of 56 700 at SinDuri and the minimum value of 9100 at YeongSangang. These values are comparable to other values measured in the area (Yoo et al. 2009a), and are slightly less than values reported for fish living in Etobicoke Creek, Ontario, which was heavily contaminated by PFCs due to a fire fighting foam spill.

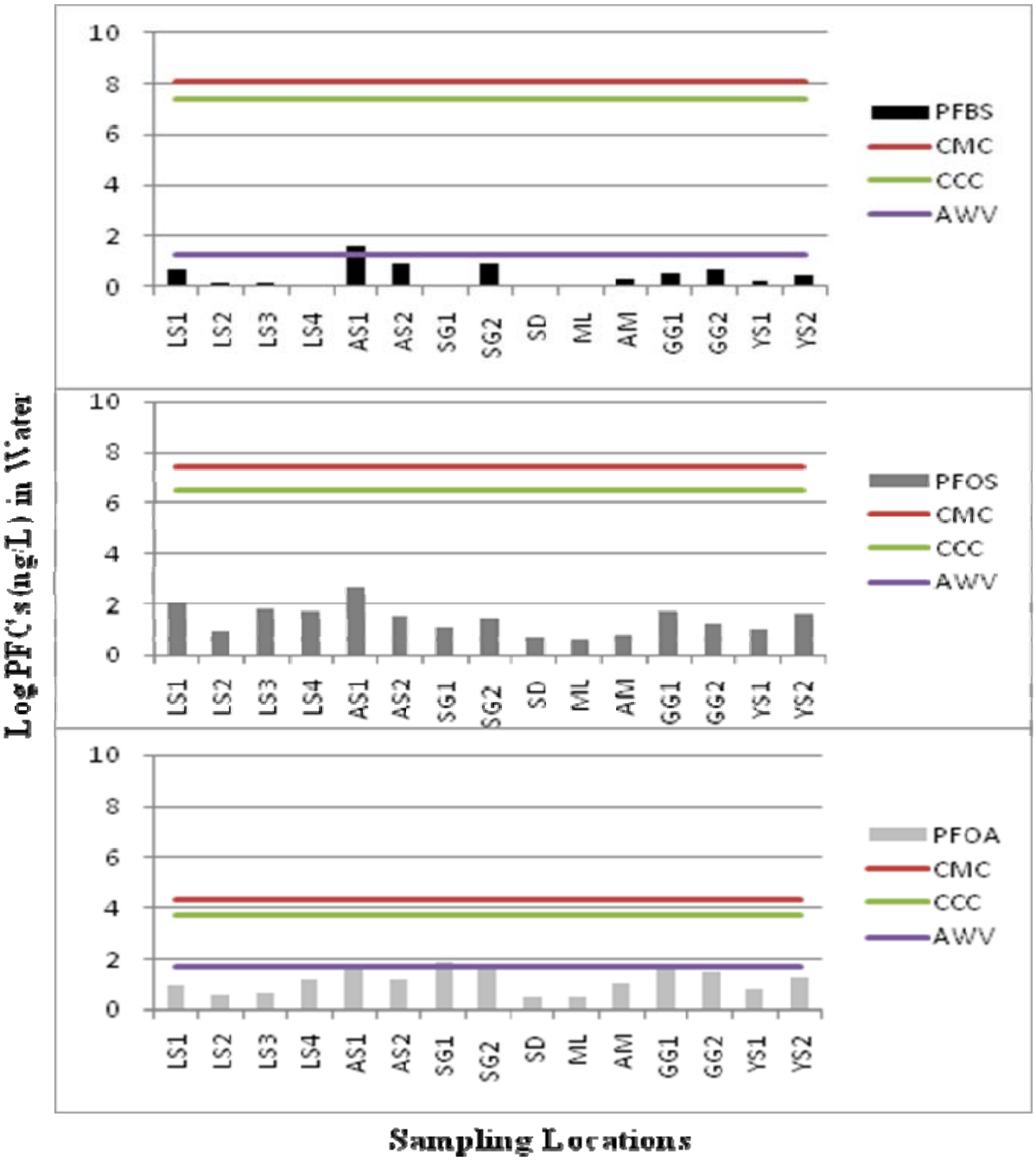
4.3.5. Potential adverse effects

Using the US EPA Great Lakes Initiative (GLI) we derived numeric water quality values for those PFCs that have sufficient and appropriate toxicity data (US EPA 1995). The GLI provides specific procedures and methodologies for utilizing toxicity data to derive water quality values that will be protective of aquatic organisms. The final acute value (FAV) is a semi-probabilistic approach that requires data for a range of specified taxa and results in a concentration that is deemed to be protective for approximately 95% of the tested genera. This FAV is used to establish a criteria maximum concentration (CMC) which is equivalent to the one-half the FAV. The criteria continuous concentration (CCC) represents a concentration of a chemical such that 95% of the genera tested have greater chronic toxicity values. The purpose and use of these numerical criteria is not to provide concentrations of a chemical that will be protective of all aquatic species in a specific ecosystem, but to provide reasonable protection to ecologically and commercially important species under most circumstance. Using modified

procedures that are explained in GLI guidance we were also able to calculate a water quality criteria for the protection of avian species (US EPA 1995).

Concentrations of PFCs in water observed in this study did not approach either the CMC or CCC values (Fig.4.4). However, concentrations of PFOS or PFOA at some locations were sufficient to potentially cause adverse effects to some wildlife at the top of the food chain, such as birds. Toxicity threshold values (TRVs) are meant to be protective and not predictive so many safety factors were included in their derivation. Thus, the actual potential for adverse effects in the most exposed species is small, but this analysis illustrates that birds have the greatest potential to be affects and could be used as sensitive and maximally exposed sentinel species for this region.

Figure 4.4 Comparison of selected PFCs (viz. PFBS, PFOS, and PFOA) in waters from the west coast of Korea with suggested water quality criteria values for the protection of aquatic organisms (CMC: criteria maximum concentration; CCC: criteria continuous concentration) and wildlife (AWV: avian wildlife value).



4.4. Conclusions

The western coast of Korea is a highly developed region of Asia that is home to millions of people and is vital for both industry and tourism alike. Previous studies found relatively high PFC pollution in Korean water but little was known about their sources, distribution and transport in a region that is known to have used PFCs extensively. As part of an ongoing study to determine the current status and extent of PFC concentrations, as well as potential for detrimental environmental effects in the Yellow Sea eco-region of China and Korea, the present study determined overall PFCs contamination from various environmental samples along the estuarine and coastal areas of Korea. Overall, our data indicated that:

- Concentrations of PFCs in estuarine and coastal areas of Korea were relatively greater than those reported in other Asian countries,
- Among 13 target PFCs measured, PFOS was consistently found at the greatest concentrations throughout the environmental media,
- Some heavier PFCs such as PFHxA, PFDA and PFUnA as well as PFOS were concentrated in biota samples, particularly in higher trophic level organisms, supporting bioaccumulation of PFCs,
- Occurrence and spatial distribution of detected PFCs in various environmental media between upstream and downstream indicated the continuing input of existing PFCs sources in Korea,
- Concentrations of PFOS or PFOA found at some locations were sufficient to potentially cause adverse effects to some wildlife, thus monitoring effort of such PFCs should be of great attention in Korea.

5 PERFLUORINATED COMPOUNDS IN WATER, SEDIMENT, SOIL AND BIOTA FROM ESTUARINE AND COASTAL AREAS OF KOREA (2009)

5.1 Introduction

Perfluorinated compounds (PFCs) have garnered increasing worldwide attention since their detection in environmental samples in 2001, due to their widespread contamination in both environmental and human samples (Giesy and Kannan 2001). Due to their amphiphilic tails and resistance to breakdown PFCs make excellent surfactants, and have been produced in relatively large quantities since the 1950s for a wide range of applications such as carpet coatings, food packaging, shampoos, paper, and fire-fighting foams (Paul et al. 2009). Some of these compounds are persistent in the environment, whereas others degrade to more environmentally stable compounds such as perfluorooctanesulfonate (PFOS), which can make determining exposure levels, and body burdens difficult (Dinglasan et al. 2004;Martin et al. 2010).

PFCs are globally ubiquitous in both remote and urban environments (Giesy and Kannan 2002) (Jin et al. 2009;Yamashita et al. 2005). PFCs are present in various matrices including; human blood (whole, plasma and serum), sediments, water, and wildlife (Giesy and Kannan 2001;Hansen et al. 2002;Higgins et al. 2004;Olsen et al. 2003b;Yamashita et al. 2005). Due to their widespread uses in many common products, PFCs are routinely found in the blood and serum of both occupationally and non-occupationally exposed people (Kannan et al. 2004;Karrman et al. 2005;Olsen et al. 2005). The most widely distributed, and also the most studied PFC is PFOS, which concentrations of PFOS in serum from occupationally exposed fluoro-chemical manufacturer employees having been shown to be as high mg/L levels in sera samples (Olsen et al. 1999).

While production of PFOS-based products was voluntarily halted by North America's largest producer; the 3M company in 2000 (3M 2000), PFOS is still being produced in relatively large quantities in China (Pan et al. 2010;Ruisheng 2008;So et al. 2007). Since the 3M Company stopped producing PFOS concentrations in human sera collected in the United States have decreased, but this trend was not uniformly observed for Asian countries, such as Korea where it appears that concentrations may in fact be increasing (Harada et al. 2010). Previous studies have reported concentrations of PFCs in Korea to be relatively high, among Asian countries, particularly high when compared to other regions around the globe (Kannan et al. 2004;Rostkowski et al. 2005;Yoo et al. 2008;Yoo et al. 2009a). The western coast of Korea is an industrialized and highly urbanized region of Asia that is home to millions of people and is vital for both industry and tourism alike. The western coast of Korean forms the eastern boundary of the Yellow Sea, which is a major commercial artery that is highly significant from both a transportation and industrial standpoint. In fact, the economic contribution of the Yellow Sea contributes about one tenth of the gross nation product for all of China (Hu et al. 2010). Recently our group showed that concentrations of PFCs found in Korean environmental samples were great enough to potentially cause adverse effects to wildlife (Naile et al. 2010). The picture of PFC pollution in Korea is becoming clearer, however questions such as yearly variation, and the distribution of PFCs found within an organism, still remain. As part of an ongoing study to determine the current status and extent of PFC concentrations, as well as potential for detrimental environmental effects in the Yellow Sea region of China and Korea, environmental samples were collected along the western coast of Korea during May of 2009. Samples were collected from similar sampling locations as were used in the 2008 study, to allow for yearly comparisons of PFCs concentrations and to detect possible changes in localized point-sources.

Concentrations of 13 PFCs in environmental samples collected from estuarine and coastal areas of Korea were determined to further assess the potential risk of PFCs to both humans and wildlife.

5.2 Materials and methods

5.2.1 Chemicals

Omni-Solv grade methanol was purchased from EMD Chemicals (Gibbstown, NJ, USA). HPLC grade ammonium acetate was purchased from J.T. Baker (Phillipsburg, NJ, USA). Sodium thiosulfate was purchased from EMD Chemicals (Gibbstown, NJ, USA). The internal standard consisted of perfluorononanoic acid (PFOA) [1,2,3,4 ^{13}C] (>98%, Wellington Laboratories), and PFOS [$^{18}O_2$] (RTI International). The external standard used for all matrix spikes was a mixture of 12 different PFCs (>98%, Wellington Laboratories) including perfluorobutane sulfonate (PFBS), perfluorohexane sulfonate (PFHxS), PFOS, and perfluorodecane sulfonate (PFDS), perfluorobutyric acid (PFBA), perfluorohexanoic acid (PFHxA), perfluoroheptanoic acid (PFHpA), PFOA, perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), perfluoroundecanoic acid (PFUnA), and perfluorododecanoic acid (PFDoA).

5.2.2 Sample collection

Water, soil, sediment, and biota were collected from 8 estuarine and coastal areas of along the western side of Korea during May of 2009 (Table 5.1, and Figure 5.1). One liter of

surface water was collected by dipping a clean, methanol rinsed one liter polypropylene (PP) bottle just under the surface of the water. Residual chlorine in each water sample was reduced by adding 200 µl of 200 mg/ml of a sodium thiosulfate solution using a disposable PP syringe. Surface (top 1-5 cm) soil and sediment samples were collected using a clean methanol rinsed stainless steel trowel. Samples were transferred and stored in clean PP bags. Biological samples were collected by hand in coastal tidal pools and along the shore of inland water bodies, and were transferred and stored in clean PP bags. Sample duplicates and field blanks were collected daily, and were analyzed along with lab and procedural blanks. All samples were transported on ice at 4°C to the laboratory and frozen at -20 °C until analyses. Biological samples were necropsied and to allowed for specific tissue analysis.

Figure 5.1 Sampling locations along the western coast of Korea, 2009

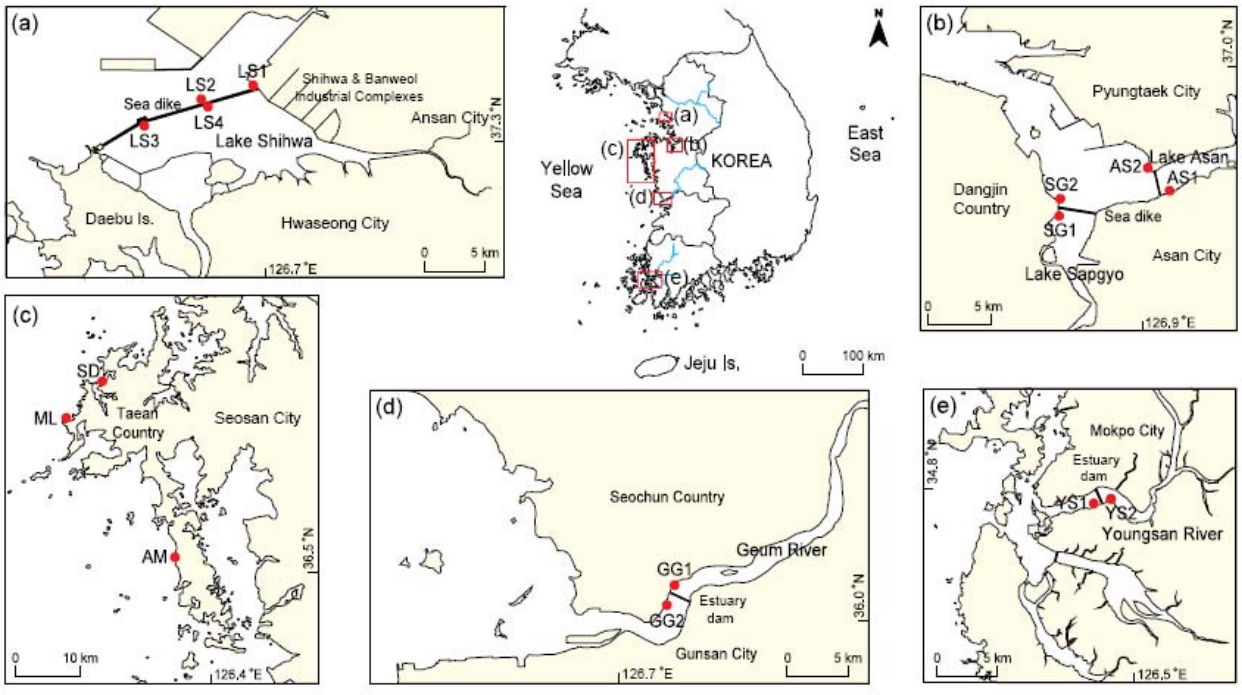


Table 5.1 Sampling details including location description and type of samples collected during the survey along the west coast of Korea

Area	Sample ID and Location		Date Collected	Soil	Sediment	Water	Biological	Field Blank
Lake **S**hihwa:	LS1	Outside of lake, Gyeonggi Bay	June-30th	O	O	O	O	O
	LS2	Outside of lake, Gyeonggi Bay	June-30th	O		O	O	
	LS3	Inside of lake				O	O	
	LS4	Inside of lake				O	O	
ASan:	AS1	Inside of lake	June-29th	O	O	O		O
	AS2	Outside of lake, Asan Bay	June-29th	O	O	O	O	
Sap**G**yo:	SG1	Inside of lake	June-29th	O	O	O	O	
	SG2	Outside of lake, Asan Bay	June-29th	O	O	O	O	
Sin**D**uri:	SD	Beach (surface)	June-28th	O	O	O	O	O
	SD'	Beach (deepth 20cm)	June-28th		O			
Man**L**ipo:	ML	Beach (surface)	June-28th	O	O	O	O	
	ML'	Beach (deepth 20cm)	June-28th		O			
AnM**y**undo:	AM	Beach (surface)	June-27th	O	O	O	O	O
Geum**G**ang:	GG1	Upstream, inside of dam	June-27th	O	O	O	O	
	GG2	Downstream, outside of dam	June-26th	O	O	O	O	O
Yeong**S**angang	YS1	Downstream, outside of dam	June-26th	O	O	O	O	
	YS2	Upstream, inside of dam	June-26th	O	O	O	O	
no. of samples				**13**	**14**	**15**	**12**	**5**

5.2.3 Extraction and cleanup

Water samples were extracted using Oasis HLB extraction cartridges (0.2 g, 6 cm^3) (Waters Corp., Milford, MA) as previously reported (So et al. 2004). In brief, the cartridges were preconditioned by eluting with 5 mL of methanol followed by 5 mL of nano-pure water at a rate of 2 drops a second. Five hundred mL of water was then spiked with 500 µL of 5 ng/mL of the internal standard (Isotopically labelled PFOS and PFOA) and then loaded onto the cartridge, at a rate of 1 drop a second, the eluent was discarded. The cartridge was then washed with 5 mL of 40% methanol in water, and the eluent was again discarded, and once complete was allowed to run dry. Lastly, the target fraction was eluted with 10 mL of methanol at a rate of 1 drop a second and collected in a 15 mL PP centrifuge tube. The resulting eluate was then reduced to 1 mL under a gentle stream of nitrogen gas, and filtered using a disposable PP syringe, fitted with a disposable PP 0.2 µm filter (Millipore, Bedford, MA, USA). Samples were stored and analyzed in PP auto-sampler vials fitted with PP septa (Canadian Life Science, Peterborough, ON, CAN), as it has been shown that glass vials and PTFE septa may cause loss of analyte and increased contamination, respectively (Yamashita et al. 2004)).

Soil and sediment samples were extracted using a previous published method by Higgins et al. with minor changes (Higgins et al. 2005). Briefly, homogenized freeze-dried 1 g samples were transferred to 50 mL PP centrifuge tubes and spiked with 500 µL of a 5 ng/mL internal standard, to which 10 mL of a 1% acetic acid solution was added. Each vial was vortexed, and placed in a heated sonication bath for 15 min. After sonication the tubes were centrifuged at 3000 rpm for 2 min and the acetic acid solution was decanted into a new clean 50-mL PP tube. 2.5 mL of a 90:10 (v/v) methanol and 1% acetic acid mixture was then added to the original vial and the vial was again vortex mixed and sonicated for 15 min, before being centrifuged and

decanted into the second tube. This process was repeated once more, and a final 10-mL acetic acid wash was preformed. All extracts were combined in the second tube before being passed through the SPE cartridge in a similar fashion as was described above in the water extraction procedure.

Biological samples were extracted using an alkaline digestion SPE method (So et al. 2006). A 1 g aliquant of homogenized freeze-dried tissue was transferred to a 50-mL PP centrifuge tube and spiked with 500 µl of 5 ng/mL internal standard, and 30 mL of 0.01 N KOH/methanol was added to the tube. The mixture was then shaken at 250 rpm for 16 hours. After this digestion 1 mL of the resulting tissue solution was added to a 1-L PP bottle containing 100 mL of nano-pure water and shaken thoroughly. This tissue-water mixture was then extracted using SPE cartridges as previously stated above.

5.2.4 Analysis

Analytical methods were optimized to allow simultaneous detection of all target analytes. Analyte separation was accomplished by use of an Agilent 1200 HPLC fitted with a Thermo Scientific Betasil C18 (100x2.1mm, 5 µm particle size) analytical column operated at 35 ^{o}C. Gradient conditions were used at 300 µL/min flow rate, starting with 60% A (2 mM ammonium acetate) and 40% B (100% methanol). Initial conditions were held for 2 min and then ramped to 20% A at 18 min, held until 20 min, decreased to 0% A at 21 min, increased to 100% A at 22 min, held until 22.5 min, returned to initial condition at 23 min, and finally held constant until 26 min.

Mass spectra were collected using an AB SCIEX 3000 (Foster City, CA) tandem mass spectrometer, fitted with an electro-spray ionization source, operated in the negative ionization

mode. Chromatograms were recorded using MRM mode, and when possible at least two transitions per-analyte were monitored (Table 2.0). The following instrument parameters were used: desolvation temperature (450 ^{o}C), desolvation (curtain) gas 6.0 arbitrary units (AU); nebulizer gas flow 5 AU; ion spray voltage – 3500 V; collision gas 12 AU; and a dwell time of 40 msec. The optimal settings for collision energies and declustering potential were determined for each analyte's transitions. Quantification using these transitions was performed using Analyst 1.4.1 software provided by SCIEX (Applied Bioscience, Foster City, CA).

5.2.5 Quality control

To reduce instrument background contamination coming from the HPLC or solvents, a ZORBEX (Thermo Scientific, 50x2.1mm, 5 um particle size) column was inserted directly before the injection-valve, as adapted from Benskin et al. (Benskin et al. 2007). Solvent blanks were run every 4 to 5 samples to check for carryover and background contamination. Concentrations of all field and solvent blanks were found to be below the limit of quantification (LOQ), where the LOQ was defined as 5x the signal measured in solvent blanks. Teflon coated lab-ware was avoided during all steps of sample and standard solution preparation to minimize contamination. The ions monitored, method detection limit (MDL), and matrix spike recoveries for all of the chemicals of interest are given (Table 5.2). The MDL was defined as the amount of chemical which could be detected in a given amount of sample after the entire analytical method was preformed.

Table 5.2 Target analytes of 12 perfluorinated compounds measured in the present study with QA/QC information including monitoring transitions, method detection limit, and matrix spike recovery for water, soil and sediment, and biological samples

Analyte	Acronym	Monitoring Transitions	Method Dection Limit			Matrix Spike Recovery		
			Water (ng/L)	Soil/Sed (ng/ml)	Biological (ng/ml)	Water (%)	Soil/Sed (%)	Biological (%)
Perfluorobutanesulfonate	PFBS	299 → 99, 80	0.2	0.2	0.2	94	32	97
Perfluorohexanesulfonate	PFHxS	399 → 99, 80	0.2	0.2	0.2	93	134	113
Perfluorooctanesulfonate	PFOS	499 → 99, 80	0.2	0.2	0.2	97	95	89
Perfluorodecanesulfonate	PFDS	599 → 99, 80	1	0.5	0.5	101	43	70
Perfluorobutanoic acid	PFBA	213 → 169	0.2	2	2	120	IS	IS
Perfluoropentanoic acid	PFPnA	263 → 219	2	2	2	82	72	79
Perfluorohexanoic acid	PFHxA	313 → 269	1	0.5	0.5	85	78	73
Perfluoroheptanoic acid	PFHpA	363 → 319, 169	1	0.5	0.5	80	123	112
Perfluorooctanoic acid	PFOA	413 → 219, 169	0.2	0.2	0.2	88	89	88
Perfluorononanoic acid	PFNA	463 → 419, 219	0.2	0.2	0.2	99	135	133
Perfluorodecanoic acid	PFDA	513 → 469, 269	0.2	0.2	0.2	89	106	75
Perfluoroundecanoic acid	PFUnA	563 → 269, 219	0.2	0.2	0.2	99	53	93

MDL was defined as amount of chemical which could be detected in a given amount of sample after the entire method was preformed

IS, Insufficient recovery

5.3 Results and discussion

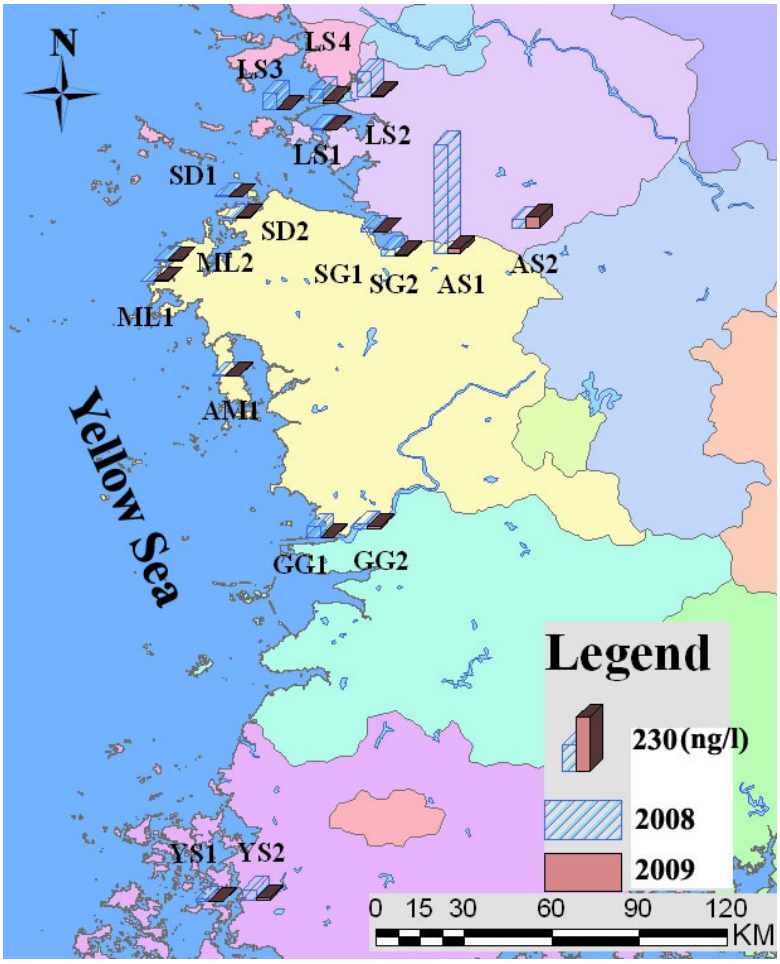
5.3.1 PFCs in water

Similarly to the 2008 samples, PFOS and PFOA were detected in all of the 2009 water samples, although the average concentration of both chemicals were found to be reduced. PFOS concentrations varied from 0.4 to 47.5 ng/L, with an average concentration of 9.1 ng/L, while PFOA concentrations varied from 0.5 to 31.4 ng/L with an average of 6.9 ng/L. Only two locations, AS2 and AM1 were found to have larger amounts of PFOS in 2009 than 2008, and PFOA was higher at only one location, which was ML1 (Figures 5.2, 5.3). Although there were great differences in the amount of PFOS and PFOA found between 2008 and 2009, the ratio of PFOS to PFOA was comparable for all but 4 of the sampling locations. The occurrence and concentrations of all monitored PFCs are summarized (Table 5.3). The specific type and amount of PFCs detected varied greatly from 2008 to 2009. In 2008 PFOS was the dominant PFC found; while in 2009 PFHpA was found at both the highest mean concentration of 15 ng/L, and the highest maximum concentration of 107.9 ng/L. In 2008, all the monitored PFCs except PFDoA were detected in at least one sample, but in 2009 there were 5 compounds that were not detected, including: PFDS, PFBA, PFPeA, PFHxA, and PFDoA. In 2009 only PFOS, PFOA were detected at all sampling locations, but both PFNA and PFDA were detected at all but one location.

Table 5.3 Concentrations (ng/ml) of PFCs detected in water samples collected from the west coast of Korea

Location	PFBS	PFHxS	PFOS	PFDS	PFBA	PFPnA	PFHxA	PFHpA	PFOA	PFNA	PFDA	PFUnA	Sum PFCs
AM	< 0.2	0.4	4.0	< 1.0	< 0.2	< 2.0	< 1.0	1.2	0.9	0.6	1.6	0.2	8.9
AS1	< 0.2	8.7	22.8	< 1.0	< 0.2	< 2.0	< 1.0	107.9	18.8	3.6	3.1	0.4	165.4
AS2	1.0	4.0	47.5	< 1.0	< 0.2	< 2.0	< 1.0	2.3	4.7	2.2	2.3	1.0	64.9
GG1	< 0.2	0.5	3.1	< 1.0	< 0.2	< 2.0	< 1.0	21.5	31.4	3.1	9.3	0.8	69.6
GG2	1.1	2.4	9.0	< 1.0	< 0.2	< 2.0	< 1.0	5.9	9.4	5.9	0.8	1.3	35.8
LS1	0.7	< 0.2	6.1	< 1.0	< 0.2	< 2.0	< 1.0	< 1.0	2.5	0.7	0.4	0.2	10.7
LS2	0.8	< 0.2	3.2	< 1.0	< 0.2	< 2.0	< 1.0	< 1.0	1.3	0.3	0.5	0.5	6.8
LS3	0.5	0.4	2.4	< 1.0	< 0.2	< 2.0	< 1.0	2.5	1.7	2.1	< 0.2	0.4	9.9
LS4	16.0	0.5	10.1	< 1.0	< 0.2	< 2.0	< 1.0	< 1.0	3.7	1.7	0.9	0.6	33.6
ML	< 0.2	0.2	0.4	< 1.0	< 0.2	< 2.0	< 1.0	1.1	0.5	1.4	3.1	0.4	6.9
SD	< 0.2	< 0.2	0.6	< 1.0	< 0.2	< 2.0	< 1.0	3.0	0.6	1.1	< 0.2	0.6	5.9
SG1	13.2	0.3	2.9	< 1.0	< 0.2	< 2.0	< 1.0	13.8	5.1	3.6	< 0.2	0.5	39.4
SG2	2.3	1.2	7.9	< 1.0	< 0.2	< 2.0	< 1.0	6.6	8.4	2.0	0.7	0.6	29.8
YS1	4.6	0.3	0.9	< 1.0	< 0.2	< 2.0	< 1.0	6.1	0.9	< 0.2	< 0.2	0.6	13.5
YS2	0.9	1.8	9.0	< 1.0	< 0.2	< 2.0	< 1.0	21.5	11.8	3.7	2.1	0.5	51.4
min	< 0.2	< 0.2	0.4	< 1.0	< 0.2	< 2.0	< 1.0	< 1.0	0.5	0.3	0.4	< 2.0	5.9
max	16.0	8.7	47.5	< 1.0	< 0.2	< 2.0	< 1.0	107.9	31.4	5.9	9.3	1.3	165.4
mean	4.1	1.7	8.7	< 1.0	< 0.2	< 2.0	< 1.0	16.1	6.8	2.3	2.4	0.6	36.8

Figure 5.2 PFOS concentrations in water samples from 2008 and 2009

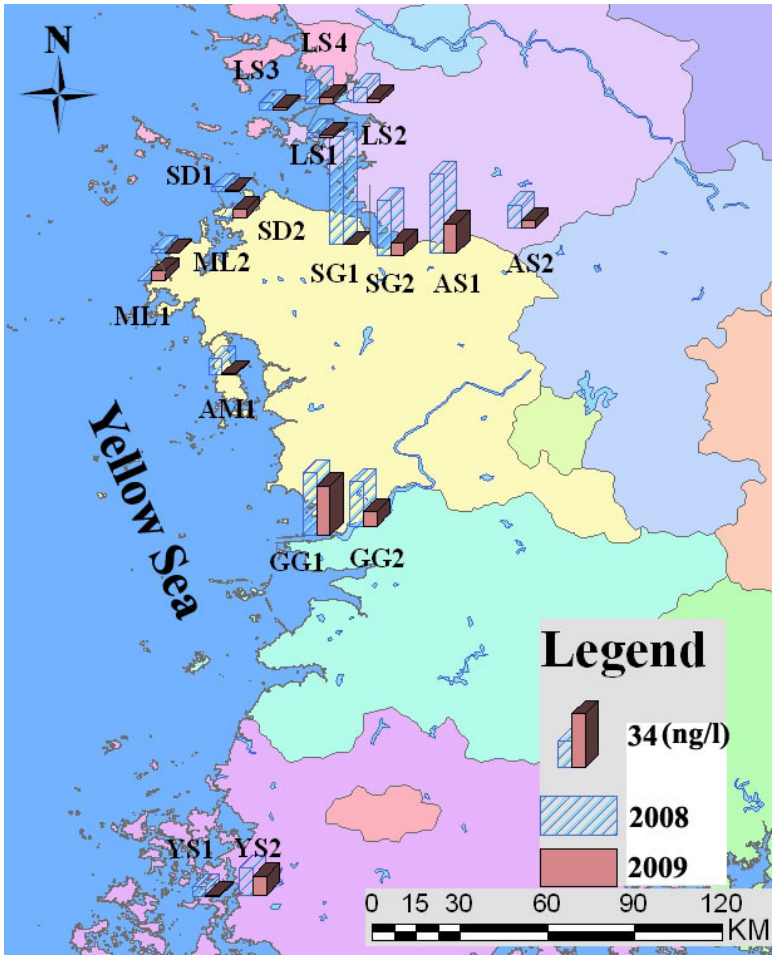


The maximum sum concentration of all PFCs was 165.4 ng/L, and was found at sampling location AS1 which is same location that produced the maximum sum concentration in 2008, although the concentration was a much higher 695.2 ng/L in 2008 (Figure 5.4). The Asan sampling locations were collected from inside (AS1) and outside (AS2) the Asan reservoir, which is adjacent to the city of Asan, which is home to many different industries. There were still large differences in the concentrations of PFCs inside and outside of the reservoir, but both concentrations were significantly reduced when compared to 2008 levels. In 2009, total PFC concentration was dominated by 3 PFCs PFHpA, PFOS, and PFOA, which had concentrations of

107.9, 22.8, and 18.8 ng/L respectively. In 2008, this location was dominated by PFOS, where as in 2009 PFHpA made up by the far the largest proportion of the total PFC concentration (Figures 5.5.1, 5.5.2). This change from predominately PFOS to predominately PFHpA was not unique to AS, but was also seen at 4 other locations. The fact that this change was observed at multiple locations throughout the sampling area, may suggest local changes in the manufacturing process of these chemicals. There were also large differences in the type of PFCs found inside and outside of the Asan reservoir. Inside the total PFC concentration was dominated by PFHpA, and to a lesser extent PFOS and PFOA. Outside of the reservoir PFOS was the dominant PFC found with PFOA and PFHxS having the next highest PFCs concentrations. This varies considerably from 2008, where both inside and outside of the reservoir PFOS was the dominant PFC found. Since the sampling was conducted at the same time in both years, differences are not seasonal, and further supports the idea of yearly changes in industrial practices.

Relatively high PFC concentrations were also seen at sampling locations GG and YS. Location GG1 was inside, and GG2 was outside of the Geumagang dam, while YS1 was outside, and YS2 was found inside of Yeongsangang dam near the city of Mokpo. The concentrations found were significantly reduced when compared to 2008 levels, but similar differences between samples collected inside of the reservoir and samples collected outside were observed, with samples collected inside being greatly elevated relatively.

Figure 5.3 PFOA concentrations in water samples from 2008 and 2009



The lowest concentrations of PFCs were found at sampling locations SD and ML which also had the lowest concentration of PFCs in 2008. Both of these locations are coastal and represent coastal ocean water levels of PFCs. These lower concentrations were also seen at the other costal sampling locations LS1, LS2, and AM. Surprisingly there was very little difference among these costal locations despite the fact that locations LS1 and LS2 are much more urban and are very close the Lake Shiwa Industrial complex which in the past has shown very high levels of PFCs (Naile et al. 2010;Rostkowski et al. 2005;Yoo et al. 2008). Lake Shiwa consists of a man-made lake, which is heavily used and influenced by more than 7,000 companies. In 2008

all of the Lake Shiwa sampling locations (LS1-LS4) had total PFC concentrations that were greater than19 ng/L and had a maximum total PFC concentration of 130 ng/L at LS1, while in 2009 only one of these locations was above 19 ng/L, which was LS4 at 34 ng/L. Since the sampling was conducted at the same time in both years, the differences seen are not seasonal and likely represent yearly changes in industrial practices. As was stated above, since the sampling was conducted at the same time in both years, differences seen are not seasonal and likely represent yearly changes in industrial practices. More studies will need to be conducted to determine whether this significant decrease in PFC water concentrations in and around Lake Shiwa is ephemeral or long-lasting.

Figure 5.4 Total PFC concentrations in water samples collected in 2008 and 2009

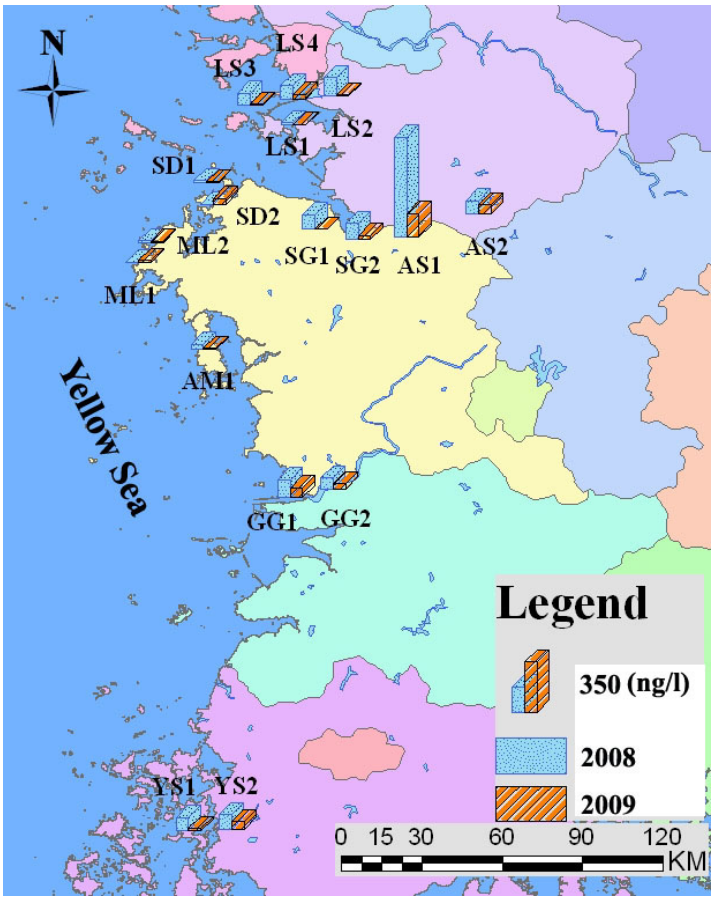
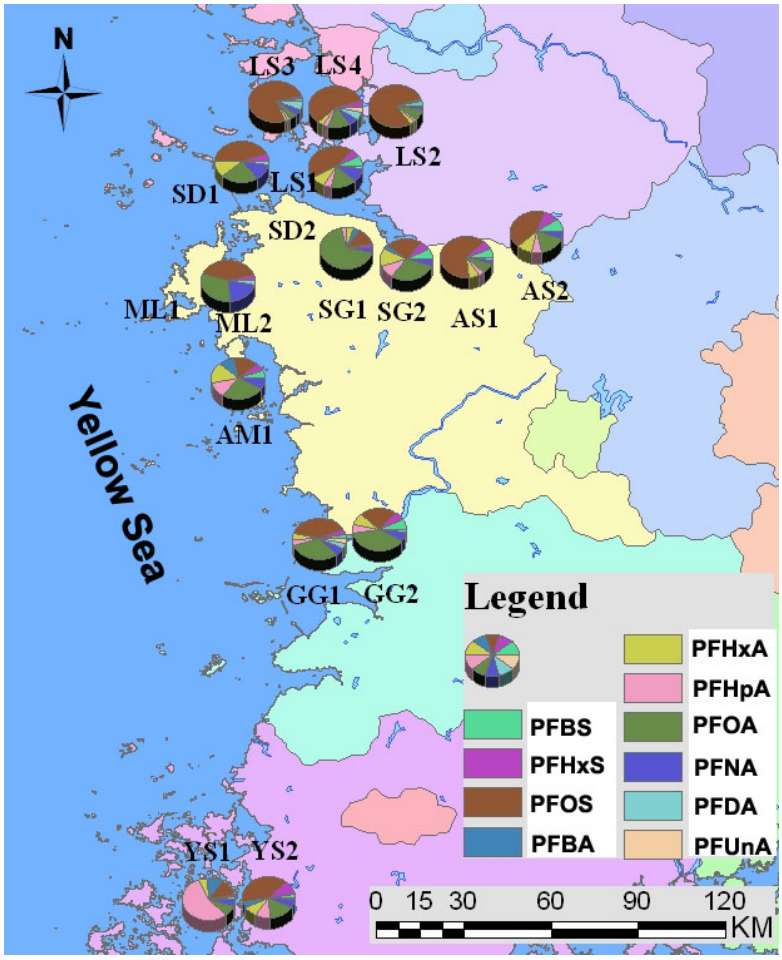
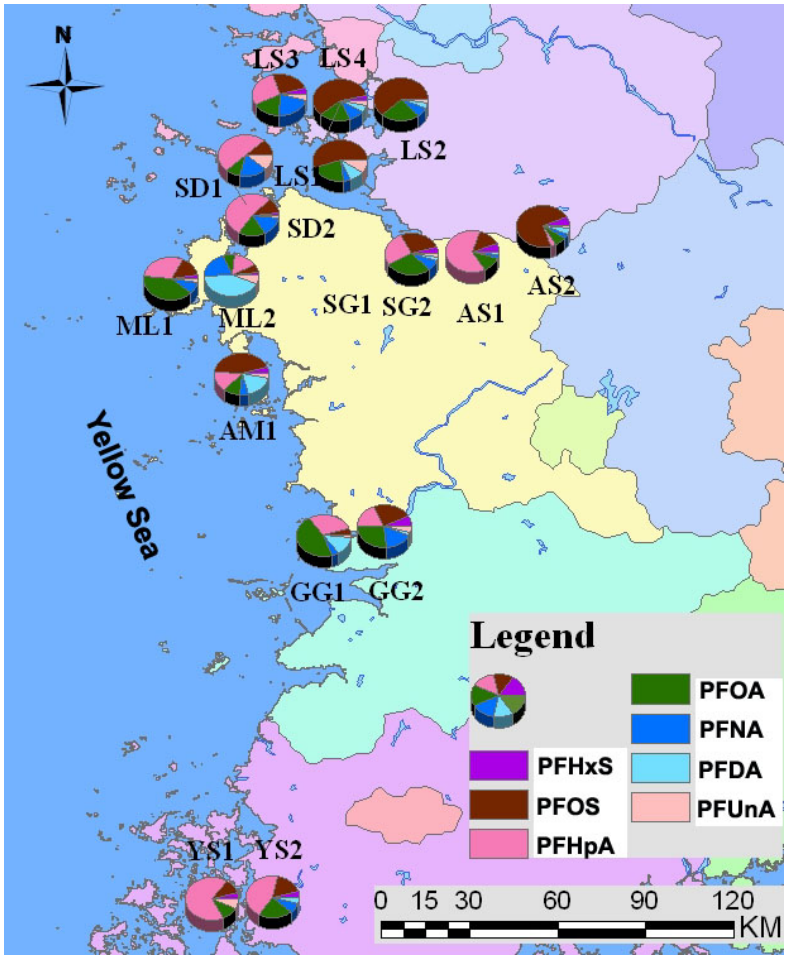


Figure 5.5.1 Percentage of individual PFCs in water samples from 2008



The concentrations of PFCs found in Korean waters in 2009 were lower than those reported previously in Korea (Naile et al. 2010;Rostkowski et al. 2005), but were still slightly elevated or comparable to other studies conducted in North America, Southern China, and open oceans. For example, a recent study also conducted on the Yellow Sea found mean PFOS and PFOA levels of 2.5 and 8.9 ng/L (n= 34) (Chen et al. 2011), which were very comparable to the 8.3 and 6.7 (n= 16) found in this study. It is still unclear if the reduced levels and differences if chemical profiles of PFCs found in water is a result of yearly differences or changes in industrial usage.

Figure 5.5.2 Percentage of individual PFCs in water samples from 2009



5.3.2 PFCs in soils and sediments

Similarly to the 2008 samples concentrations, of PFCs in soils and sediments were generally less than the MDL, and when detected were generally less than those measured in biota. Concentrations were similar to those previously reported in other areas of Asia, and slightly less than have been reported in Europe and the United States (Bao et al. 2009;de Voogt and Van Roon 2005;Higgins et al. 2005;Nakata et al. 2006). Recently Pan et al. found very high concentrations of PFOS in sediments collected from the mouth of the Yangtze River, while Bao

et al found relatively low levels of PFOS and PFOA only slightly upstream on a tributary of the Yangtze (Pan and You 2010). This suggests that the importance of sediments and soils as a sink for PFCs is highly variable and dependent on both environmental conditions and the source of the input. PFOS and PFOA were detected in 7 and 3 of 19 sediment samples, and in only 3 and 2 of 19 soil samples, respectively. There did seem to be slight preference for the detection of PFOS and PFOA in sediments as opposed to soils, but when detected, the concentrations in soils and sediments did not vary significantly between the two matrices. As was seen in the previous year's study, it appears that soil and sediment samples collected along the western coast of Korea contain small amounts of PFCs and do not appear to a contribute significantly to the exposure of benthic or terrestrial organisms.

5.3.3 PFCs in biota

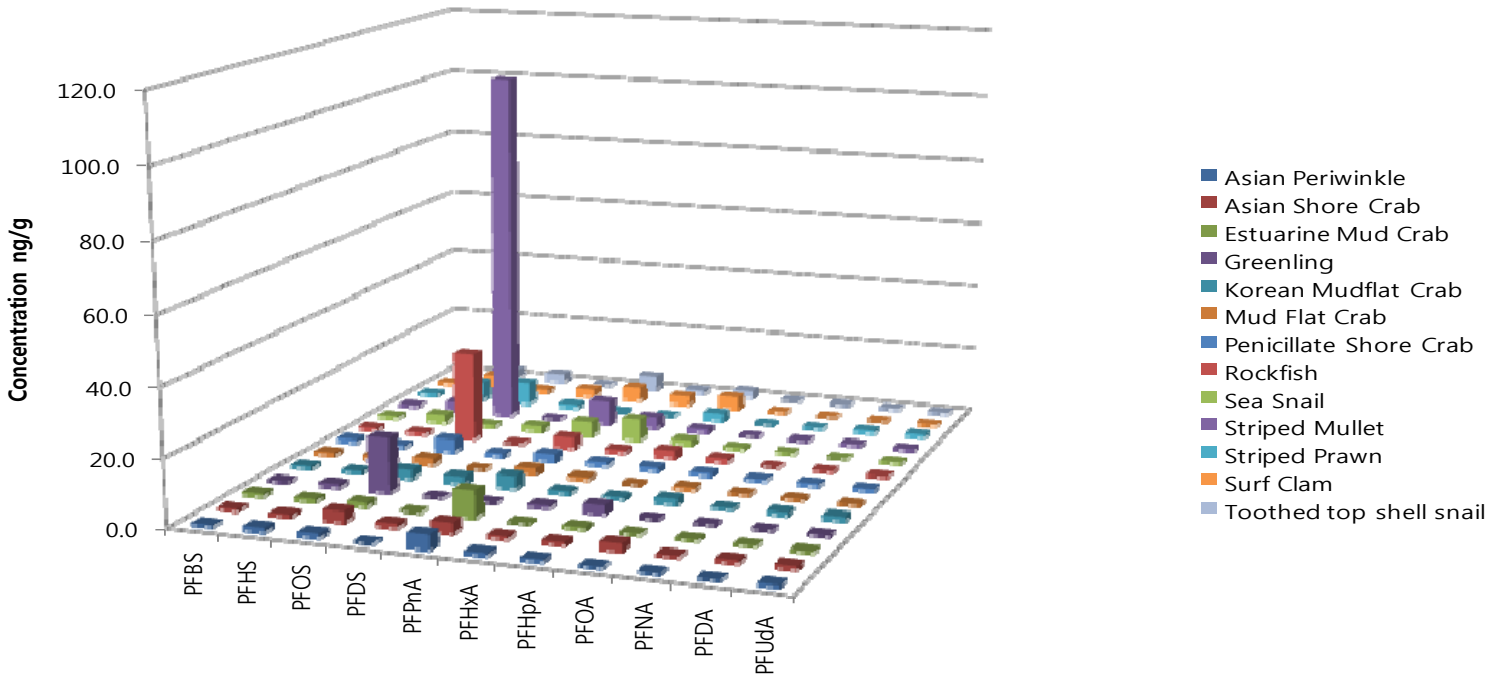
Similarly to what was observed for water and sediment, concentrations of PFCs in biota samples from 2009 were smaller than were measured in 2008. PFOS was once again found to be the dominate PFC found in biota with a maximum concentration of 229.8 ng/g dw, and a mean concentration of 13.9 ng/g dw. Although PFOS was again found to be the dominant PFC there were large differences in the relative percent of each PFC found in biological samples. With the exception of crustaceans, the relative percent of PFOS significantly decreased in the four groups of biological samples analyzed. In general, 2009 samples were highly variable and less homogenous than 2008 samples, with PFCs such PFBS, PFHS, and PFPnA making up a larger proportion. PFOS was found above the MDL in 71 of 76 biological samples, where as in 2008 PFOS was detected in all of the 21 samples. PFPnA was found to have the next highest mean

concentration of 4.9 ng/g dw, and had a maximal concentration of 26.5 ng/g dw. Similarly to 2008, PFOA was found at low levels with a maximum concentration of 5.3 ng/g dw and average of 0.6 ng/g dw. The highest levels of PFOS were all found in fish tissue, with two of the top three concentrations coming from location YS1, whereas the largest amount of PFPnA was found in a mussel (*Mytilus edulis)* from location LS4. In 2008, the highest level of PFCs were found in fish from locations SD and YS. Although the amounts detected from these locations differed significantly from 2008 to 2009, it is clear that high concentrations of PFCs persist at these locations and that some organisms are concentrating these chemicals.

When comparing PFC concentrations among the different biological species sampled, it is clear that PFOS is by far the most dominant chemical present and that fish accumulate PFCs to a much greater extent than any of the other organisms sampled (Figure 5.6). In fact, the 3 fish species sampled had the 3 highest concentrations of PFOS of any biological samples collected. PFPnA was also routinely found, but it pattern of detection/amount did not match well with what was observed for PFOS, suggesting that the two chemicals most likely come from different sources.

To further understand how biological samples were accumulating PFCs, organisms were necropsied to allow individual tissue determination. The body distribution for both crabs and fish are shown in Figures 5.7 and 5.8 respectively. PFOS and PFPnA were dominant across all tissue types, and followed a similar pattern with the body have the highest concentration, followed closely by the shell, and the legs containing the least. The relative amount found in each sample type varied among the different chemicals tested, but in general the amount found in the legs was lower than either the shell or body. This could be useful information for regulators, as the most

Figure 5.6 Concentrations of PFCs in various biological species



commonly consumed part of a crab are its legs and they appear to have the lowest amount of PFCs among the different tissue types. The body distribution for the fish sampled was much less variable than what observed for crabs, with PFOS being the dominant PFC detected across 3 of the 4 tissue types. PFOS was found to be the highest in intestinal tissue, which matches what was observed in the previous year's study where very great concentrations of PFOS were found intestinal tissue (Naile et al. 2010). The liver was found to have the next greatest amount of PFOS, which is not surprising as PFOS is routinely detected at high concentrations in the liver of aquatic organisms (Kannan et al. 2001a;Kannan et al. 2002b;Martin et al. 2004b). The average fillet concentration of PFOS was about 30 ng/g, which was approximately 8 times less than what was found in the intestines. This finding could be great use to regulators as the fillet is the most commonly consumed part of a fish, and this shows that the use of whole body concentrations to set safe consumption guidelines would not be accurate.

Figure 5.7 Body distribution of PFCs in crabs collected from Korea

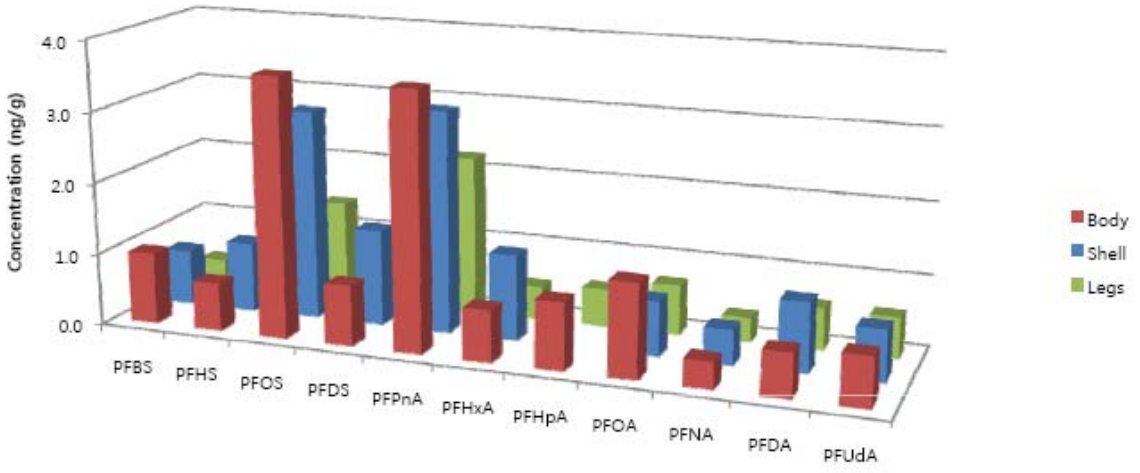
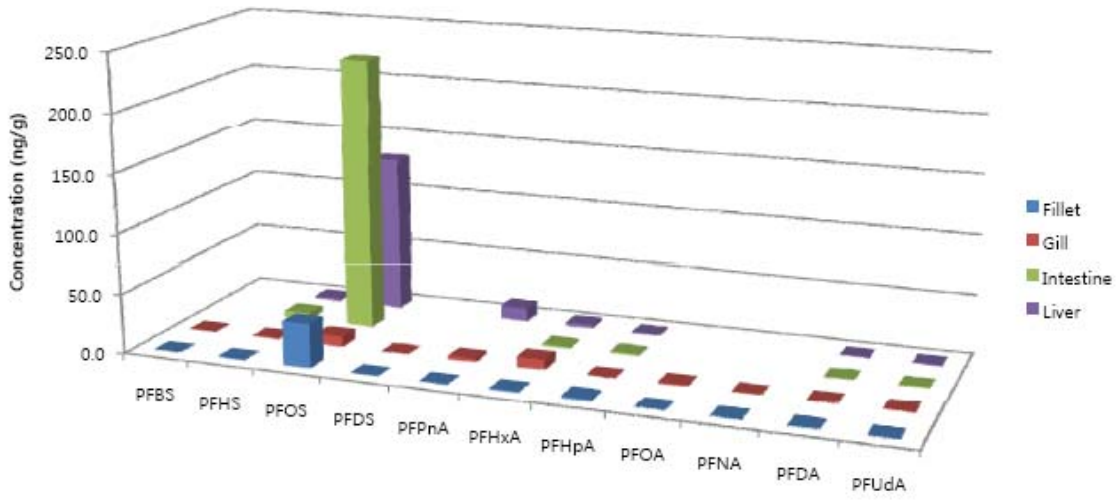


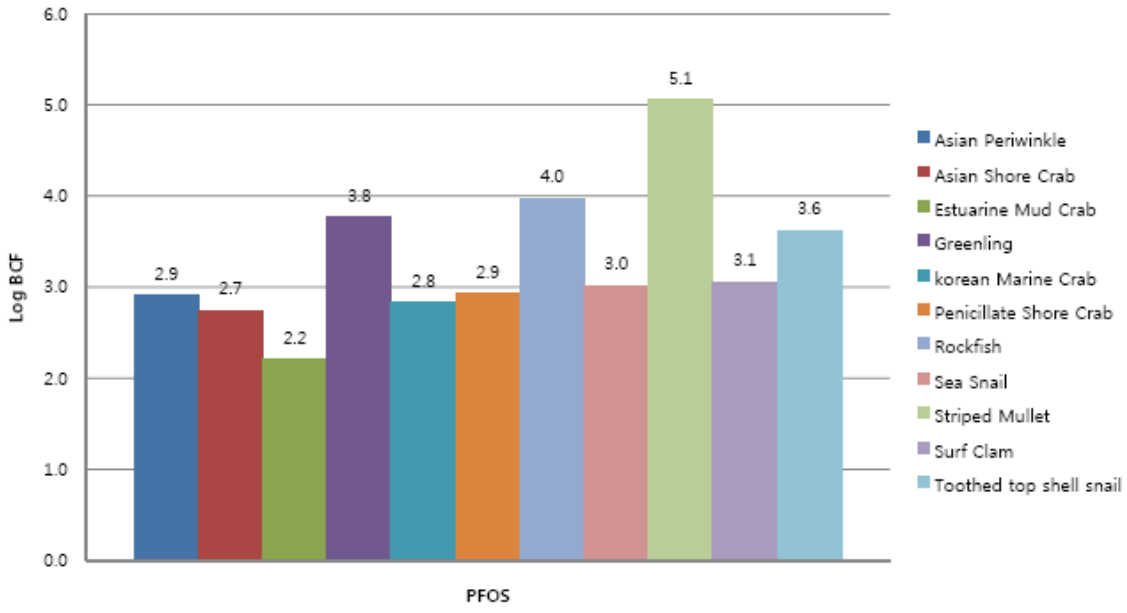
Figure 5.8 Body distribution of PFCs in fish collected from Korea



Field-based bioconcentration factors (BCF) were calculated for aquatic organisms using site specific PFOS water concentrations in the estuarine and coastal areas of the western Korea. A field-based BCF was defined as the concentration in the organism divided by the concentration in water. The mean BCF values for all species was 12,555 with a maximum value of 113,502 for stripped mullet and minimum value of 159 for the estuarine mud crab. Log BCF values for 11 species are shown in Figure 5.9. The organisms with the second and thrid highest BCFs were the rockfish and greenling respectfully, and in general the observed BCF for fish were much higher than in other other organisms such as crabs. The field-BCFs calculated in this study were higher for fish and comparable for crabs, to what was previously observed in Korea. Hoon et al. calculated a mean BCF for mullet to be between 3,700-12,400, and a BCF for Blue crab to be aproximatley 575, where the BCFs calucted in this study were 113,502 and 159-852 for mullet and crabs respectively (Yoo et al. 2009b). The calculated BCFs in this study were also comparable to a recent study in China also conducted around the Yellow Sea, where BCFs in

fish ranged from 3,100 to 85,000 (Wang T. et al. 2011). The BCFs presented here are among the highest ever reported for fish, and are comparable to the values reported for Etobicoke Creek, Ontario following a spill of aqueous fire-fighting foam (log BCF of 3.8-5.1) (Moody et al. 2002). Like what is often the case the field-BCFs in this study were much higher than what has been reported in lab based studies (Giesy et al. 2010). This discrepincy between field and lab based BCFs has not been well explained and needs further attention to allow for accurate risk assesment.

Figure 5.9 BCFs for PFOS in biological samples from Korea



5.4 Conclusions

As part of an ongoing study to determine PFCs concentrations, distributions, sources and the current environmental condition of the Yellow Sea ecosystem, the present study has provided detailed anaylsis of yearly variation and specific tissue distribution of PFCs. The current findings

should help researchers and regulators better understand the extent of PFCs contamination and the current risk to both humans and wildlife, in the estuarine and coastal areas of the western coast of Korea. Further detailed work and continued long term intensive sampling efforts would help to determine the reasons for observed yearly deferences and whether concentrations are infact, decreasing in the region.

6 EFFECTS OF PERFLUORINATED COMPOUNDS ON GENE EXPRESSION OF THE H4IIE RAT HEPATOMA CELL LINE

6.1 Introduction

Due to their perfluorinated carbon tail and polar head group, PFCs are excellent at repelling both water and oil and are used as surfactants in many applications (Kissa 2001). PFCs have been produced and used in relatively large quantities since the 1950's, for a wide range of applications ranging from carpet coatings, food packaging, paints, and fire retardants (Paul *et al.,* 2009). Although many PFCs have surfactant properties, the eight-carbon-long derivatives are the most effective for several applications. It is these same useful properties imparted by the carbon-fluorine bond that causes these compounds to be resistant to metabolic and environmental degradation, and as a result it is the eight-carbon length chemicals that are often the most accumulated and toxic. PFCs are ubiquitous in air water, sediments, soils, wildlife and human blood and milk from remote as well as urban environments where they are manufactured, used, and disposed of (Chen et al. 2011;Giesy and Kannan 2001;Masunaga et al. 2002;Yamashita et al. 2005).

There are two main classes of PFCs; the sulfonates which have a sulfonate head group attached to the C-F backbone, and carboxlyates which have a carboxclyic-acid group attached to the backbone. The most widely distributed, and also the most studied PFC is PFOS. Perfluorooctane sulfonate, which consists of 8 carbons and 17 fluorine atoms was one of the most useful PFCs, but was also found to be one of the most persistent and toxic (Renner, 2006). As a result, production of PFOS-based products was voluntarily halted in 2000 by North America's largest producer; the 3M company (3M, 2000).

The toxicology of PFOS and PFOA has been extensively reviewed (Kennedy, Jr. et al. 2004;Lau et al. 2007). In non-human primates and rodents PFOS exposure causes reduced body weight, increased liver weight, reduced cholesterol synthesis, and has a steep dose-response curves for mortality (Butenhoff et al. 2002;Luebker et al. 2002;Seacat et al. 2003). Exposure to PFOS causes thyroid follicular cell adenomas in rats (Chang et al. 2008;Chang et al. 2009;Seacat et al. 2003). Both PFOS and PFOA have been shown to cause similar toxicological effects, but PFOS is known to be an incomplete peroxisome proliferator, whereas as PFOA has been shown to be a potent, complete peroxisome proliferator (Lau et al., 2007). Exposure to PFOA has also been shown to cause liver, Leydig cell, and pancreatic acinar-cell tumours in rats (Kennedy, Jr. *et al.,* 2004).

While the toxicity and mechanisms of action of PFOS and PFOA have been studied and thresholds for effects determined, few studies of the toxicity of shorter-chain replacement chemicals, such as PFBS and PFBA have been conducted (Das et al. 2008;Lieder et al. 2009;Newsted et al. 2008). Replacement chemicals have many of the same useful chemical properties, but are less accumulated in animals, and have significantly shorter half-lives in humans and rodents (Olsen et al. 2009). While less potent, these chemicals have been shown to act through similar mechanisms as PFOS (Newsted et al. 2008;Wolf et al. 2008). Since shorter chain-length PFCs are generally less toxic, and do not accumulate to the same extent as PFOS, the 3M Company has been using the C4 analogue PFBS as a replacement for the C8 PFOS in products such as Scotchgaurd® (Renner, 2006).

The effects of PFCs on mRNA expression have been examined in a variety of animal's including birds, fish, and rats (Hickey et al. 2009;Hu et al. 2005b;Shi et al. 2008). These studies have shown that mRNA expressions of several classes of genes are commonly affected by PFCs.

Specifically, mRNA expression of genes related to peroxisome proliferation, gap-junction communication, thyroid development, fatty acid metabolism, cholesterol synthesis, and lipid transport have been identified as being impacted by PFCs. Although these pathway-based findings support previous findings, most of these studies have been performed using just PFOS or PFOA, and few studies have reported on alterations in mRNA expression in response to other PFCs (Kennedy *et al.* 2009, Wolf *et al.,* 2008).

This current study examined the effects of 10 PFCs routinely found in the environment on 7 different genes related to processes known or hypothesized to be affected by PFOS. These processes include fatty acid synthesis, cellular communication, and thyroid gland development. The objective of this study was to utilize changes in the mRNA abundance caused by exposure to PFCs to determine whether PFCs of varying lengths and functional groups cause similar cellular responses to the model PFCs, PFOS and PFOA. To test the null hypothesis that all PFCs, including shorter chain-length replacements, act via similar modes of action, rat H4IIE cells were exposed to 10 PFCs, all of which are routinely found in the environment, and the mRNA abundance of 7 target genes was quantified using a real-time PCR.

6.2 Materials and Methods

6.2.1 Chemicals and Standards

Omni-Solv grade methanol was purchased from EMD Chemicals (Gibbstown, NJ, USA). 10 different PFCs were donated by the 3M company including PFBA (99.2%, Sigma-Aldrich), PFPnA (99.2%, Alfa Aesar), PFHxA (97.7%, Oakwood Products), PFOA (ammonium salt, 95.2 %, Lot # 332), PFNA (98%, Oakwood Laboratories), PFUdA (96.4%, Oakwood Laboratories),

PFDoA (99.65%, Oakwood Laboratories), PFBS (97.3%, 3M Lot #2), Perfluorohexane sulfonate PFHxS (98.6%, 3M NB 120067-69), and PFOS (potassium salt, 86.9%, Lot #217).

6.2.2 Cell culture and Exposure

Cells were cultured and treated similarly to the methods described by (Hu et al. 2005a). Briefly, H4IIE rat hepatoma cells between the 5^{th} and 10^{th} passage were cultured in 100 mm disposable tissue culture dishes at 37°C under sterile conditions in a humidified 5/95% CO_2/air incubator. Cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Cells were removed from the dish with a trypsin/EDTA solution and split into tissue culture plates at a seeding density of $1x10^6$ cells/mL. Cells were then incubated for 24 h to allow attachment to the new plate. After 24 h; the medium was replaced with medium containing the PFCs of interest to achieve final concentrations of 0.1, 1, 10, or 100 μM. The solvent control was 0.01% methanol and the blank control was medium only. Cells were incubated with the exposure media for 72 h. Following the exposure period the medium was removed, and cells were harvested by adding 200 ul of trypsin and gently agitating the plates at 37°C for 5 min. Cells were then transferred to 600 μL micro-centrifuge tubes and centrifuged for 30s at ≥10,000 rpm. The supernatant was removed and the pellet was stored at -80 °C until analysis.

6.2.3 RNA Isolation and first-strand cDNA synthesis

Total RNA was isolated from cells using the RNeasy® Plus Mini Kit (QIAGEN, Mississauga, ON, Canada) according to the manufacturer's recommended protocol. RNA concentrations were determined at A_{260} using a ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). First strand cDNA was synthesized from 1 μg of total RNA using an

iScript cDNA Synthesis kit (BioRad, Mississauga, ON, Canada) with an oligo dT primer according to the manufacturer's instructions.

6.2.4 Real-Time PCR

Changes in mRNA expression of target genes and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (G-3-PDH) were measured by quantitative real-time PCR (qPCR) using SYBR green on an ABI 7300 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). PCR reaction mixtures (20 µl) contained 1 µl of forward and reverse primers (Invitrogen, Burlington, ON, Canada), 1 µl of cDNA, 8 µl of nuclease free water, and 10 µl of 2 X SYBR Green^{TM} PCR Master Mix (Applied Biosystems). The thermal cycle profile was as follows: denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation for 15 s at 95 °C and annealing with extension for 1 min at 60 °C. Melt curve analyses were performed on all samples to ensure amplification of a single PCR product. All reactions were performed in triplicate. All primers were used at the same final concentration and sequences of the primers used to amplify target mRNAs are shown in table 6.1.

6.2.5 Data and Statistical Analysis

Relative changes in mRNA expression of PFC treated cells relative to solvent treated control cells were calculated according to Pfaffl (Pfaffl et al. 2002). All data were checked for equal variances and normality, using the Levene and Kolmogorov-Smirnov tests respectively. Non-parametric statistics were performed using an ANOVA type Kruskal-Wallis and a post hoc pair-wise Mann-Whitney U test. All statistical analyses were performed using the statistical software SYSTAT® 17.0 Package (SYSTAT Software Inc., Richmond, CA), with a significance level of $p < 0.05$.

Table 6.1 List of the genes examined, and their corresponding primer sequences and accession numbers

Gene Name	Acronym	Direction	Sequence (5' - 3')	Accession Number
Glyceraldehyde-3-Phosphate Dehydrogenase	G3PDH	Forward	ATGACTCTACCCACGGCAAG	M17701
		Reverse	GGAAGATGGTGATGGGTTTC	
Paired box gene 8	PAX8	Forward	CTACCTAGCCTCTGCCAACG	BC081779
		Reverse	TGGAGCAGTTGCCTAGTGTG	
Homeobox	Hex	Forward	ACTACACGCACGCCCTACTC	BC088135
		Reverse	GCCTTTCCTTTTGTGCAGAG	
Apolipoprotein A-IV	ApoA4	Forward	GAGGGTGAGGGAAGAGATCC	NM012737
		Reverse	TGTTCCTGCAACTTCTGCAC	
Squalene synthase	SqSyn	Forward	ACTCCCCCATTTACCTGTCC	M95591
		Reverse	ACCCACTTCACGTGGACTTC	
Peroxisome 3-ketoacyl-CoA thiolase	Per-3Keto-Alpha	Forward	AGGAAAACCATCACCGTGTC	J02749
		Reverse	TGCTCCATCACTCACCTGAC	
Mitochondria 3-ketoacyl-CoA thiolase	Mito-3Keto-Alpha	Forward	AACCTCCAAGGACACCACAG	D16478
		Reverse	TCTGACATCATGGGAGCAAG	
Mitochondria 3-ketoacyl-CoA thiolase	Mito-3Keto-Beta	Forward	AAGCCTGCATTCATCAAACC	D16479
		Reverse	TTATAACCCATGGCCAGAGC	

6.3 Results

6.3.1 Cell Viability

Cell viability was assessed visually at the time of seeding and at the end of the exposure. The viability of cells treated with 0.01% methanol did not differ from the viability of untreated cells and cell viability was not compromised by any of the chemicals at any of the concentrations tested.

6.3.2 Effects of PFOS and PFOA on mRNA Abundance

The mRNA abundance of the reference gene glyceraldehyde-3-phosphate dehydrogenase (*G3pdh*) did not differ between the non-exposed cells, the solvent control cells, and PFC exposed cells. The fold change in mRNA abundance for the 7 genes in response to each of the 10 chemicals is shown (Table 6.2). Values in dark grey indicate a significant increase while values in light grey indicate significant decrease in mRNA expression.

PFOS and PFOA had similar effects on the mRNA abundance for only 2 of the 7 genes tested (Table 6.3). Expression of peroxisome 3-ketoacyl-CoA thiolase (*Per-3-Keto-α*), showed the greatest difference between the two chemicals. Specifically, exposure to PFOA up-regulated *Per-3-Keto-α* mRNA abundance by approximately 7-fold whereas exposure to PFOS down-regulated *Per-3-Keto-α* mRNA expression by approximately 5-fold. Large differences between the responses caused by exposure to PFOS versus PFOA were also seen for the homeobox (*Hex*) gene; however, differences were only in the magnitude of mRNA abundance and not direction. A dose-response was only observed for mRNA abundance of mitochondria 3-ketoacyl-CoA thiolase (*Mito-3-Keto-α)* (Figure 6.1). Therefore, effects summarized are based on the average change in mRNA expression for the 4 concentrations.

Table 6.2 Mean fold change ± standard error of mRNA expression. Means and standard error were calculated based on data from 4 replicates. Dark grey values indicated significant increase in fold change whereas light grey values indicate a significant decrease in fold change, relative to the solvent control (P< 0.05)

Chemicals											
	Conc. (uM)	**Gene**	**PFBS**	**PFHS**	**PFOS**	**PFBA**	**PFPnA**	**PFHA**	**PFOA**	**PFNA**	**PFUdA**	**PFDoA**
Average Fold Change	**100**	***Mito-3-Keto-α***	0.31	0.80	0.48	0.12	1.09	0.64	0.54	1.99	0.32	1.46
STD ER			0.03	0.08	0.08	0.02	0.12	0.09	0.06	0.31	0.04	0.18
Average Fold Change	**10**	***Mito-3-Keto-α***	0.22	0.73	0.27	0.08	0.31	0.45	0.27	1.04	0.26	0.68
STD ER			0.04	0.11	0.08	0.03	0.04	0.10	0.01	0.13	0.01	0.01
Average Fold Change	**1**	***Mito-3-Keto-α***	0.17	0.81	0.26	0.06	0.61	0.53	0.65	0.99	0.13	0.51
STD ER			0.02	0.24	0.09	0.02	0.10	0.10	0.23	0.14	0.03	0.11
Average Fold Change	**0.1**	***Mito-3-Keto-α***	0.22	0.74	0.28	0.11	0.59	0.52	0.42	0.91	0.22	0.52
STD ER			0.06	0.05	0.05	0.03	0.10	0.11	0.14	0.15	0.02	0.16
	Conc. (uM)	**Gene**	**PFBS**	**PFHS**	**PFOS**	**PFBA**	**PFPnA**	**PFHA**	**PFOA**	**PFNA**	**PFUdA**	**PFDoA**
Average Fold Change	**100**	***ApoA4***	0.56	0.67	1.93	1.89	0.72	2.18	1.03	4.36	1.41	2.24
STD ER			0.15	0.13	0.67	0.11	0.13	0.42	0.25	1.20	0.14	0.33
Average Fold Change	**10**	***ApoA4***	0.14	0.33	0.25	1.61	0.35	5.25	1.99	2.40	0.80	0.55
STD ER			0.03	0.09	0.04	0.13	0.08	1.18	0.19	0.47	0.19	0.08

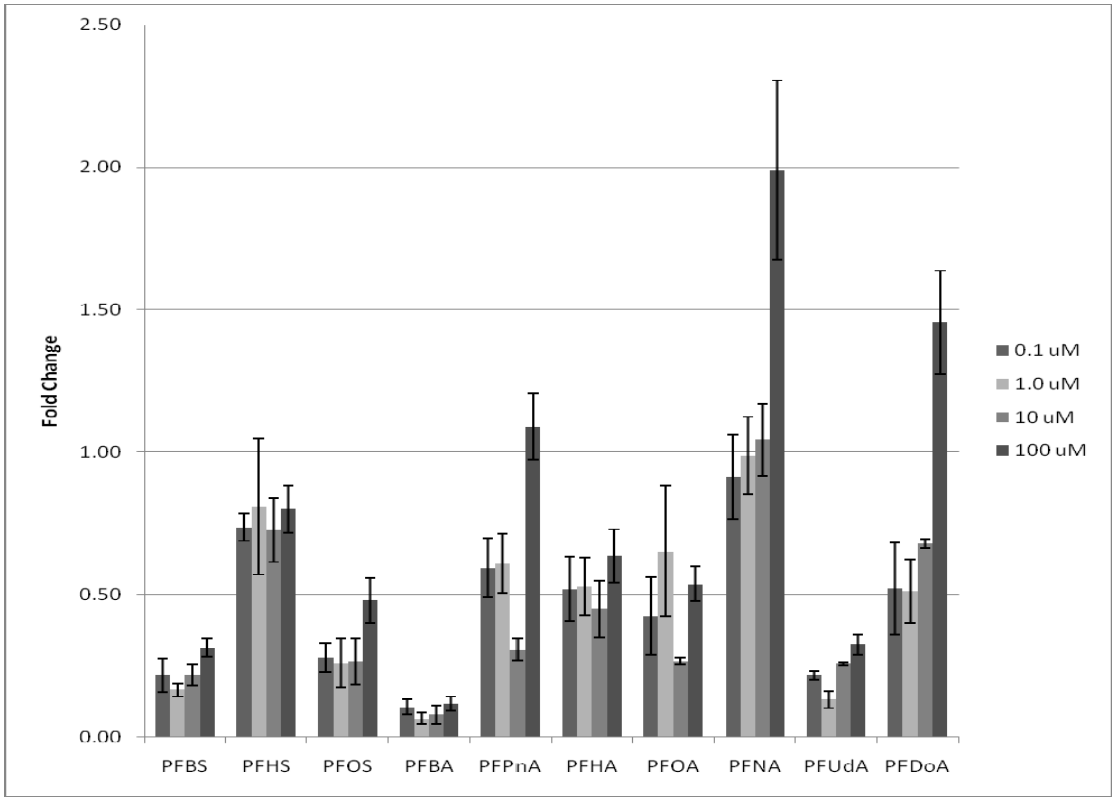
Average Fold Change	**1**	***ApoA4***	0.07	0.51	0.55	0.59	0.32	1.47	0.43	2.51	1.02	0.29
STD ER			0.02	0.16	0.15	0.07	0.08	0.64	0.02	0.54	0.29	0.07
Average Fold Change	**0.1**	***ApoA4***	0.09	0.50	0.64	0.67	0.38	3.30	0.31	3.84	1.10	0.51
STD ER			0.03	0.10	0.14	0.17	0.09	0.80	0.09	0.80	0.16	0.13
	Conc. (uM)	**Gene**	**PFBS**	**PFHS**	**PFOS**	**PFBA**	**PFPnA**	**PFHA**	**PFOA**	**PFNA**	**PFUdA**	**PFDoA**
Average Fold Change	**100**	***Per-3-Keto-α***	0.25	24.23	0.26	0.11	4.55	2.46	7.16	0.84	0.63	3.12
STD ER			0.07	6.50	0.03	0.02	0.85	0.25	2.13	0.07	0.13	0.34
Average Fold Change	**10**	***Per-3-Keto-α***	0.31	22.10	0.19	0.13	2.26	4.51	3.94	0.39	0.62	2.58
STD ER			0.08	4.13	0.04	0.04	0.16	1.08	0.46	0.05	0.04	0.30
Average Fold Change	**1**	***Per-3-Keto-α***	0.16	16.81	0.17	0.11	3.35	5.34	7.35	0.49	0.49	2.11
STD ER			0.04	3.55	0.05	0.02	0.64	1.35	0.84	0.14	0.12	0.42
Average Fold Change	**0.1**	***Per-3-Keto-α***	0.38	18.51	0.14	0.07	4.65	5.56	8.85	1.03	0.49	2.22
STD ER			0.01	1.32	0.04	0.01	1.84	0.85	0.65	0.37	0.09	0.31
	Conc. (uM)	**Gene**	**PFBS**	**PFHS**	**PFOS**	**PFBA**	**PFPnA**	**PFHA**	**PFOA**	**PFNA**	**PFUdA**	**PFDoA**
Average Fold Change	**100**	***Mito-3-Keto-β***	1.98	3.05	0.56	0.44	4.47	1.48	1.53	2.55	2.95	10.60
STD ER			0.31	0.34	0.11	0.17	0.60	0.30	0.17	0.27	0.24	0.93

Average Fold Change	**10**	***Mito-3-Keto-β***	1.29	2.12	0.24	0.28	1.31	1.33	1.07	2.24	2.33	4.40
STD ER			0.20	0.31	0.08	0.07	0.15	0.26	0.27	0.20	0.40	0.33
Average Fold Change	**1**	***Mito-3-Keto-β***	1.18	1.86	0.29	0.18	1.46	1.72	1.71	7.77	1.40	2.15
STD ER			0.17	0.58	0.09	0.08	0.20	0.53	0.34	0.49	0.35	0.40
Average Fold Change	**0.1**	***Mito-3-Keto-β***	1.33	4.27	0.27	0.20	2.69	3.22	1.05	13.15	2.02	2.47
STD ER			0.17	0.11	0.08	0.04	0.34	1.67	0.14	1.24	0.34	0.21
	Conc. (uM)	**Gene**	**PFBS**	**PFHS**	**PFOS**	**PFBA**	**PFPnA**	**PFHA**	**PFOA**	**PFNA**	**PFUdA**	**PFDoA**
Average Fold Change	**100**	***SQSYN***	0.43	14.81	0.38	0.76	1.67	14.96	1.84	21.15	0.87	31.50
STD ER			0.04	2.37	0.08	0.07	0.27	2.83	0.30	3.22	0.14	3.72
Average Fold Change	**10**	***SQSYN***	0.33	8.87	0.18	0.94	0.78	11.36	0.81	9.00	0.54	6.15
STD ER			0.11	1.46	0.05	0.10	0.08	2.14	0.03	0.66	0.03	0.11
Average Fold Change	**1**	***SQSYN***	0.24	10.35	0.23	1.12	0.36	12.14	0.92	13.41	0.47	10.90
STD ER			0.04	3.17	0.09	0.20	0.03	2.62	0.05	2.98	0.15	0.36
Average Fold Change	**0.1**	***SQSYN***	0.42	15.67	0.18	0.71	0.71	23.75	0.97	27.42	0.52	15.79
STD ER			0.17	3.25	0.04	0.08	0.15	4.70	0.21	3.45	0.12	4.81
	Conc. (uM)	**Gene**	**PFBS**	**PFHS**	**PFOS**	**PFBA**	**PFPnA**	**PFHA**	**PFOA**	**PFNA**	**PFUdA**	**PFDoA**

Average Fold Change	**100**	***Hex***	1.14	25.17	2.15	0.81	16.03	23.80	22.37	6.33	1.70	11.67
STD ER			0.18	0.22	0.61	0.28	2.39	3.62	3.51	0.77	0.36	2.86
Average Fold Change	**10**	***Hex***	1.11	16.43	3.01	0.67	4.15	19.10	16.53	2.87	1.03	6.35
STD ER			0.21	3.37	0.36	0.15	0.07	0.39	0.06	0.94	0.11	0.53
Average Fold Change	**1**	***Hex***	0.52	24.38	2.70	0.83	7.07	27.90	22.49	1.53	0.82	5.60
STD ER			0.05	0.80	0.92	0.16	1.92	5.74	2.97	0.32	0.13	0.33
Average Fold Change	**0.1**	***Hex***	1.00	29.05	2.81	0.56	7.58	29.39	33.33	1.86	7.78	4.02
STD ER			0.22	7.56	0.52	0.13	0.64	4.74	6.96	0.12	1.78	0.20
	Conc. (uM)	**Gene**	**PFBS**	**PFHS**	**PFOS**	**PFBA**	**PFPnA**	**PFHA**	**PFOA**	**PFNA**	**PFUdA**	**PFDoA**
Average Fold Change	**100**	***PAX 8***	4.80	11.98	1.40	6.78	2.20	3.73	4.19	2.20	1.29	12.21
STD ER			0.42	3.74	0.40	3.30	0.30	0.28	0.68	0.54	0.40	2.37
Average Fold Change	**10**	***PAX 8***	1.27	7.85	0.21	3.55	0.50	1.80	12.82	1.92	0.88	7.41
STD ER			0.01	0.39	0.07	1.07	0.17	0.42	5.74	0.57	0.19	0.09
Average Fold Change	**1**	***PAX 8***	0.61	5.79	0.23	3.49	1.00	0.92	2.19	2.47	2.46	1.65
STD ER			0.14	0.48	0.07	NA	0.35	0.10	0.23	0.77	0.61	0.38
Average Fold Change	**0.1**	***PAX 8***	0.56	4.84	0.17	1.14	1.53	2.46	1.52	16.99	1.62	4.50

STD ER	0.16	0.65	0.05	0.53	0.15	0.44	0.20	11.11	0.41	3.23

Figure 6.1 Concentration dependent affects of 10 PFCs on the expression of Mito-Keto-α after a 72h exposure. Means and standard error were calculated based on data from 4 replicates (N=4)



6.3.3 Effects of Sulfonates on mRNA Abundance

PFOS and PFBS caused similar effects on mRNA abundance of all target genes, except for slight differences in expression of *Hex* and mitochondria 3-ketoacyl-CoA thiolase (*Mito-3-Keto-β*) (Table 6.2). The mRNA abundance of *Hex* was significantly increased by 2.5-fold in cells exposed to PFOS, while PFBS caused no significant change in *Hex* transcript abundance. *Mito-3-keto-β* mRNA abundance decreased 3-fold in cells exposed to PFOS, but there was no change in transcript abundance in cells exposed to PFBS. Similar effects of PFBS and PFOS on the mRNA abundance of apolipoprotein A-IV (*ApoA4*) were observed. Exposure to PFBS

caused a significant decrease in expression in 3 out of 4 of the doses tested, and had an average fold-change of about 11, whereas PFOS-treated cells were only affected at one of the 4 doses. However, PFOS and PFBS had similar effects on the mRNA abundance of squalene synthase (*Sqsyn*). Both PFOS and PFBS caused an average decrease in mRNA abundance of 4-fold across all doses. PFHS had different effects on the mRNA abundance of 5 of the 7 target genes compared to PFOS or PFBS (Figure 6.2). The three sulfonates caused similar effects on the mRNA abundance of only two genes, *ApoA4* and *Mito-3-Keto-α*. The greatest changes in mRNA abundance were consistently caused by PFHS with average changes greater than 20 fold for both *Hex* and *Per-3-Keto-α*.

6.3.4 Effects of Carboxylates on mRNA Abundance

The effects of PFOA and PFBA on mRNA adundance were similar for 5 of the 7 genes studied, but significant differences in mRNA abundance of *Per-3-Keto-α* and *Hex* were observed. The responses to the carboxylates tested varied greatly in direction and magnitude of response (Figure 6.3). In general, the effects caused by PFOA were rather random when compared to other carboxylates, and no clear chain length-dependent effects were observed.

Figure 6.2 Fold changes in target genes caused by exposure to perfluoroalkyl sulphonates for 72h. Means and standard error were calculated based on data from 4 replicates (N=4)

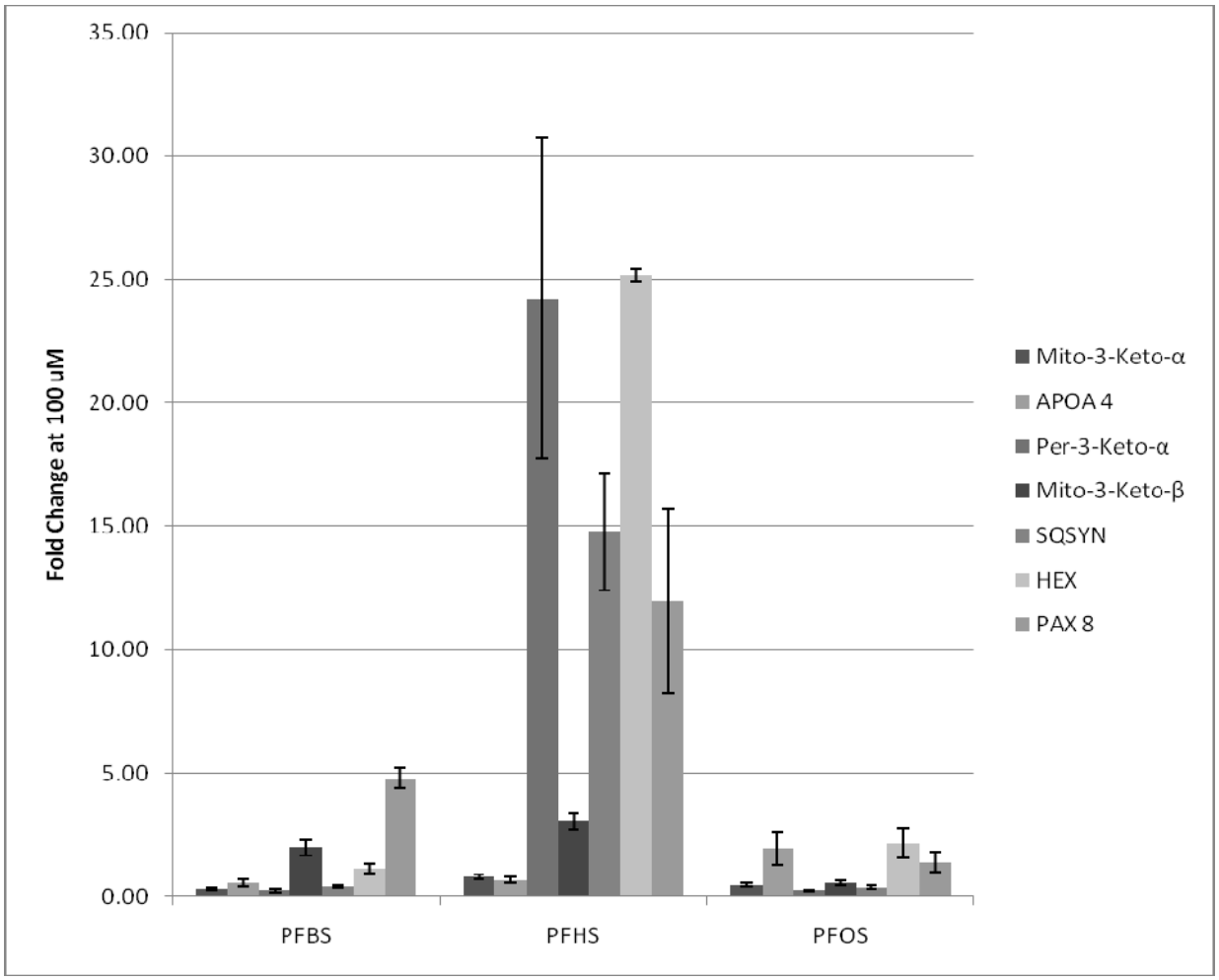
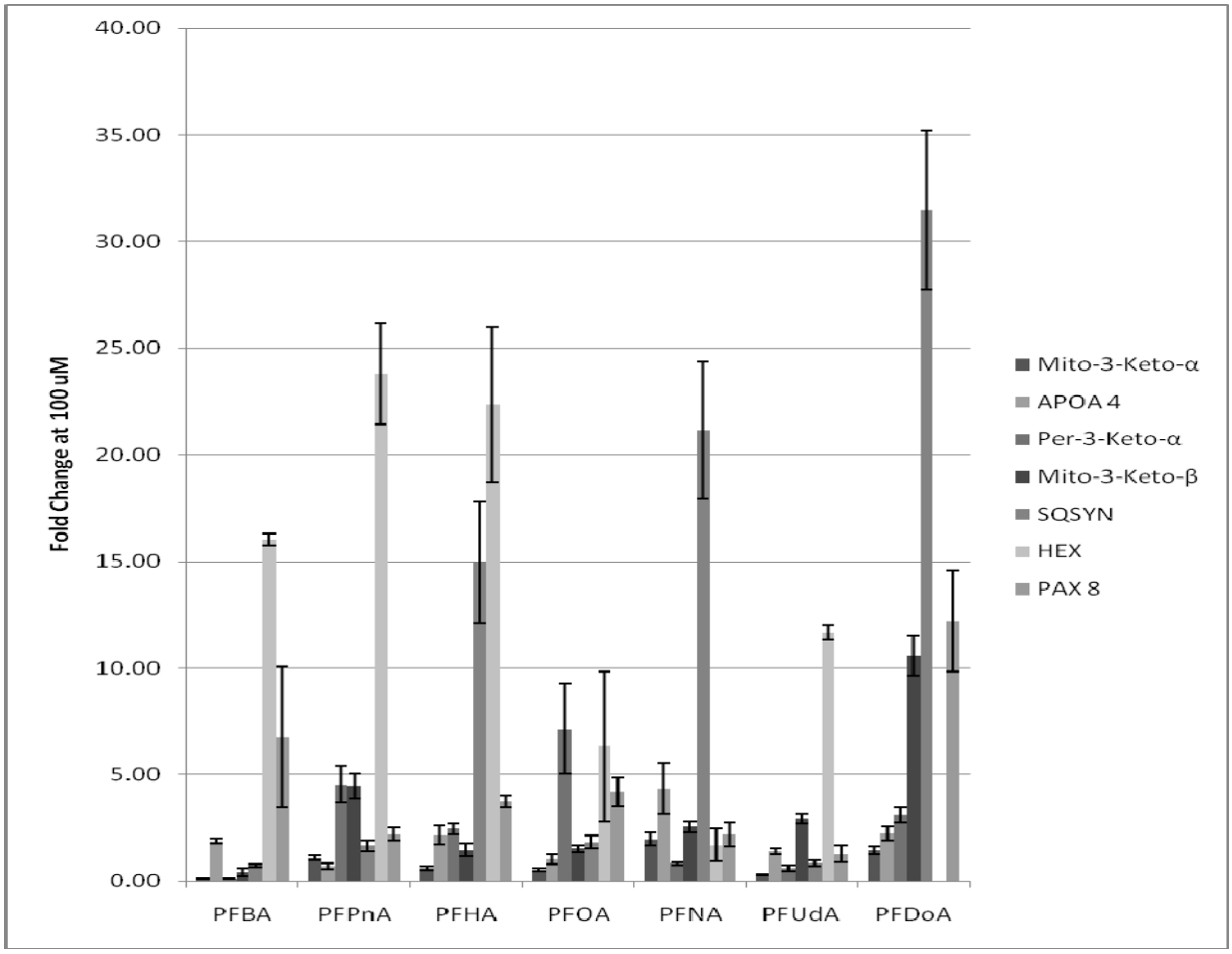


Figure 6.3 Fold changes in target genes caused by exposure to perfluoroalkyl carboxylates for 72h. Means and standard error were calculated based on data from 4 replicates (N=4).



6.4 Discussion

Production of PFOS-based products was voluntarily halted in 2000 by North America's largest producer, the 3M company (3M 2000). However, replacement chemicals which have many of the same useful chemical properties as PFOS, but have shorter half-life's and are less bioaccumulative, continue to be produced and used in large quantities. While they are believed to be less toxicologically potent, these replacement chemicals are thought to work through similar mechanisms as PFOS and/or PFOA. However, few studies have investigated this

hypothesis (Newsted *et al.,* 2008; Wolf *et al.,* 2008). As these replacement PFCs are used in commerce and released into the environment it will be necessary to assess their potential toxicity, including their precursors and terminal degradation products. To do this, a toxic equivalency approach that would allow the normalization of different PFCs based on their relative potencies has been suggested (Yoo et al. 2008). Several studies have reported on the effects of PFCs other than PFOS and PFOA on biologically relevant endpoints, including mRNA expression (Foreman et al. 2009), but very few have compared the effects of a wide variety of PFCs (Wolf *et al.,* 2008, Hickey *et al.,* 2009), and none have focused on possible thyroid related effects. To this end, H4IIE rat hepatoma cells were used to compare the effects of 10 PFCs on mRNA abundance of several genes known to be responsive to PFOS.

Currently, it is unknown whether effects caused by PFOS are indicative of effects caused by other sulfonated PFCs. Expression of several genes has been recently reported to be significantly different for PFOS than some of the other sulfonated PFCs (Hickey *et al*., 2009). In the current study, with the exception of the greatest dose, PFOS and PFBS caused different effects on the paired box gene 8 (*Pax 8*) mRNA abundance, and exposure to PFHS caused significantly greater responses than PFOS in 5 of the 7 genes studied. This finding was both unexpected and significant since it is often assumed that if PFOS does not cause an effect, then sulfonates of shorter chain-lengths would not elicit an effect (Bjork et al. 2008).

Previous studies have demonstrated effects of PFCs on thyroid hormone and thyroid related processes (Mascia *et al.,* 2002;(Shi et al. 2008). The genes chosen to potentially demonstrate differences in responses caused by various PFCs were *Pax 8* and *Hex*, because it has been shown that PFOS caused significant up-regulation of these genes in exposed zebra fish (*Danio rerio*) (Shi *et al.,* 2008). *Pax 8* is a member of the PAX family of transcription factors

and is involved in thyroid development and in the proliferation and differentiation of thyroxine-producing follicular cells (Mascia *et al.,* 2002). It has been demonstrated that *Pax 8* is significantly elevated in developing zebrafish embryos exposed to small concentrations (≤ 1 mg/ml) of PFOS (Shi *et al., 2008)*. Abundance of *Pax 8* mRNA in cells exposed to PFHS was greater than both PFBS and PFOS, with exposure to PFHS resulting in a significant up-regulation of *Pax 8* at all doses, with an average change of approximately 8-fold.

The homeobox genes, including *Hex*, encode a family of transcription factors that plays a vital role in cell differentiation during development (Gehring, 1987), including early thyroid development (Thomas *et al.,* 1998). Significant up-regulation of *Hex* in zebrafish exposed to PFOS has been demonstrated by Shi *et al*. (2008). The mRNA abundance of *Hex* was significantly different between cells exposed to PFOS and PFBS, and similar differences were also seen when comparing between cells exposed to PFOA versus PFBA. In both cases the magnitudes of the responses in cells exposed to the replacement chemicals were less than those in cells exposed to PFOS or PFOA. This suggests that PFOS and PFOA may be conservative predictors for effects of replacement chemicals.

Previous *in vivo* studies have shown that PFCs alter processes related to cholesterol and fatty acid related processes (Kudo et al. 1999;Sohlenius et al. 1993). The mRNA abundance of 5 genes associated with fatty acid and cholesterol related processes were quantified in order to compare the affects of PFCs other than PFOS and PFOA. Squalene synthase is a key enzyme in the cholesterol synthesis pathway. As such, alterations in its expression can cause adverse affects to the entire cholesterol synthetic pathway, which can reduce levels of low density lipoprotein (LDL) cholesterol, and ultimately could impact other related physiological processes, including sex hormone steroidogenesis (Roy *et al.,* 2009). The mRNA abundance of *Sqsyn* in

cells exposed to PFBS and PFBA was similar to concentrations in cells exposed to their respective model compounds, PFOS and PFOA. Taken together, it appears that for this gene both PFOS and PFOA serve as good predictors for the effects of their analogous C4 replacements.

Great differences in the mRNA abundance of *Per-3-keto-α* were observed in cells exposed to the different PFCs. Five compounds caused significant up-regulation of *Per-3-keto-α*, and the other 5 caused significant down-regulation. The largest changes were observed for PFHS which at the greatest dose caused an almost 25-fold increase in *Per-3-keto-α* mRNA levels, with similar results being observed at all of the concentration tested. This was different from the effects observed for the other 2 sulfonates, as PFOS and PFBS caused significant down-regulation of mRNA abundance, with average fold changes or approximately 4- and 6-fold respectively.

All of the carboxylates tested had a similar effect on *Pax 8* mRNA abundance, and agreed reasonably well with PFOA. This may imply that each of the carboxylates effect expression of this gene through a similar mode of action. The direction of the fold change was consistent between PFOA and PFDoA, but the large differences in magnitude may reflect the beginning of some chain length-dependent effects. PFBA and PFOA caused similar changes, and both exhibited comparable dose response effects. To the authors knowledge this is the first study to examine the response of thyroid related genes to cells exposed to perfluorinated carboxylates, so it is difficult to draw comparisons, but it is clear the expression of these genes is affected, and the responses are not always comparable in either magnitude or direction of response.

Plasma lipoprotein metabolism is regulated and controlled by specific apolipoproteins that are vital to transport and redistribution of lipids among various cells and tissues (Mahley *et al., 1984*). Recently an epidemiological study has shown a positive correlation between PFC exposure and lipoprotein concentrations (Steenland et al. 2009). Three carboxylates caused consistent significant changes in *ApoA4* mRNA expression. PFHA and PFNA caused significant up-regulation whereas PFPnA caused consistent down-regulation. PFBA and PFOA followed a similar pattern and showed minimal changes in expression.

Differences in the mRNA abundance of *Mito-3-Keto-β* suggest possible chain-length-dependent effects of carboxylates. All of the PFCs with more than 8 carbons significantly increased expression, whereas compounds of chain-length C8 or less did not. This same trend was not observed for the sulfonates, which suggests that PFCs with different functional groups may have different potency for this specific gene. Differences in mRNA abundance of *Mito-3-Keto-β* were also apparent in cells exposed to PFOS and PFOA compared to PFBS and PFBA. There was no effect of PFBS on transcript abundance but significant down regulation effects were observed for in 3 of the 4 PFOS treatments. In contrast, PFOA exposure caused no significant effects, whereas PFBA caused significant down regulation of mRNA abundance. These responses further support the hypothesis that not all PFCS behave similarly at the genomic level, and that caution should be used when making estimates based only on model PFCs, such as PFOS or PFOA.

The greatest fold change in mRNA expression for the gene *Mito-3-Keto-α* was observed in cells exposed to PFBA. This is different from the results of most other studies, whether toxicological or genomic in nature, in which PFBA was observed to cause significantly reduced effects than PFOA (Wolf *et al.,* 2008; Hickey *et al.,* 2009; Das *et al.,* 2008). Both PFOS and

PFOA behaved similarly and caused an approximate 4-fold decrease in mRNA expression for the gene *Mito-3-Keto-a*. This further highlights the point that effects cannot simply be attributed to chain-length or functional group, and that in the case of studies of mRNA abundance, the target gene may be the most important factor to consider.

6.5 Conclusions

The results of this study illustrate the utility of the quantification of mRNA abundance as a relatively simple high-throughput method of classifying similar chemicals into different toxicological groups, based both on the direction and magnitude of response. The data presented here clearly shows that not all PFCs cause the same effects on gene expression and that effects seen could not simply be attributed to chain-length or functional group. Furthermore, the responses seen for many of the shorter chain-length replacement chemicals differed significantly from the model compounds PFOS and PFOA. PFCs with chain-lengths of C6 or less are currently widely used and mass produced, and the differences seen between the different chemicals could mean that for some genes these replacement chemicals do not act through the same mechanisms as their model compounds. The differences shown here demonstrate that not all PFCs are equipotent as modulators of mRNA abundance and that making predictions simply based on chain-length or functional group may not be appropriate for many mechanisms. Specifically, if these differences between PFHS and the other sulfonates exist at more toxicological relevant endpoints, than current federal regulations regarding these chemicals may need further review.

7 SUMMARY DISCUSSION, CONCLUSIONS, AND FUTURE WORK

Perfluorinated compounds, which have been produced in relatively large quantities and used for a wide range of applications such as carpet coatings, food packaging, shampoos, paper, and fire-fighting foams since the 1950s were only recently found to occur in the environment and humans. Because of their relatively new discovery and their unique physical-chemical properties, many analytical, environmental, and toxicological questions still remain. This dissertation is the result of a series of studies designed answer some of these questions, by addressing several explicit, testable null hypotheses and bridge some of the knowledge gaps that are currently preventing accurate environmental risk assessment. But to answer these questions a robust, sensitive and accurate method of analysis for a range of PFCs was needed, that is the first thing that I developed. I then went on to apply that method to survey a number of environments to answer the questions of what PFCs are in the environment and at what concentrations. I was able to use a mass balance approach to determine when I had identified all of the PFCs accounting for the organically bound fraction of fluorine in the environment. Finally I addressed the question of who cares by working out, by the use of modern techniques of molecular biology to determine the mechanisms f action and toxic potencies of a range of the most prevalent PFCs from the environment and those that are being used as replacements for those that are being phased out. In the end I was able to develop a fairly comprehensive picture that could be used by regulators to establish environmental policy. As a result of my research, to date about 87 individual precursor chemicals have been banned in Canada and the results of my research bring into question the methods used to establish that the currently used replacements may not be as safe as thought when I began my studies. So my results that were just recently published are likely to have a further impact on environmental policy here in Canada and around the world.

In the course of my research, I worked on a number of aspects of environmental chemistry and toxicology, here in North America and in Asia. The results of those studies have been reported in 16 papers published in the peer-reviewed, open literature. Here in the dissertation, I have been able to include just a few examples of those results.

7.1 Principal Findings

Before being able to address the questions that I, and Pfor. Giesy and government regulators and scientists in industry had, I had to develop a method to identify and quantify individual PFCs in a wide range of matrices. It was shown that depending on the sources, both PFOS and PFOA standards might contain significant amounts of impurities, which could potentially cause a significant over-estimation of many environmentally relevant PFCs. It was also shown that not all reported purities of standards are necessarily accurate and that the choice of suppliers is therefore, very important. When the response factors of isotopically-labeled and native standards were compared, and it was observed that not all labeled analytes have the same response factor as their non-labeled complements, and that depending on the matrix, there may be significant suppression or enhancement of response, relative to a methanol based standard. In general, these results show that unless great care is taken to ensure proper standard selection and use, researchers may generate data that could be significantly inaccurate. This means that there is a high probability that concentrations of PFCs reported in the literature (in particular early papers) that did not use isotopically-labeled matrix-matched calibration curves many be inaccurate and should be thoroughly reviewed before being used for risk assessment proposes.

The findings from my analytical study were made immediately useful in my second and third studies which set out to determine the current status trends of concentrations of PFCs in the environment and the extent of their distribution, as well as the potential for detrimental environmental effects in the Yellow Sea eco-region of Korea and China. Concentrations of PFCs in estuarine and coastal areas of Korea and China were relatively greater than those reported in other Asian countries, and among 13 target PFCs measured, PFOS was consistently found at the greatest concentrations throughout the environmental media. The occurrence and spatial distribution of detected PFCs in various environmental media between upstream and downstream indicated the continuing input of existing PFCs sources in Korea. In some cases, concentrations of PFOS or PFOA were sufficient to potentially cause adverse effects to wildlife, thus making monitoring studies of PFCs of great importance.

PFCs have been found at detectable concentrations throughout the world, and the results from the Korean and Chinese environmental assessment demonstrate that PFCs other than the predominant PFCs, PFOS and PFOA, are routinely found, and due to the phase-out of PFOS may be making up an increasingly greater proportion of the entire PFCs load found in the environment. Hence, it is important to not only understand the mechanism of toxic action of PFOS and PFOA, but also how the other commonly detected PFCs do as well. The data presented here demonstrates that not all PFCs cause the same effects on gene expression and that effects seen could not simply be attributed to chain-length or functional group. Furthermore, the responses seen for many of the shorter chain-length replacement chemicals differed significantly from the model compounds PFOS and PFOA. PFCs with chain-lengths of C6 or less are currently widely used and mass produced. The differences seen between the various chemicals could mean that for some genes these shorter chain-length replacement chemicals do not act

through the same mechanisms as their model compounds. The differences shown here demonstrate that not all PFCs are equipotent as modulators of mRNA abundance and that making predictions simply based on chain-length or functional group may not be appropriate for many mechanisms.

The data and conclusions generated from the above studies are of great use to both scientists and regulators alike. Analytical methods have been validated and improved, and standard operating procedures have been in put in place to ensure that any new data generated will be accurate. The PFCs are a difficult set or compounds for which to analyse. This is why there were not detected in the environment until very recently. Once it was known that this "new" class of residues was present in the environment, my people started to analyze for them, but due to a lack of methods and authentic standards progress was slow and many of the results that were published were inaccurate. This more accurate and precise data will allow regulators to make better, more accurate policy, concerning the production and use of PFCs. Proper policy and regulation is important for any country but in places where elevated concentrations have been found, it is imperative to ensure environmental safety.

PFCs are ubiquitous in the environment, and laboratory studies have shown that they are toxic to a wide range of species. Concentrations found in locations such as South Korea are approaching threshold levels where adverse effects may be seen. PFOS was routinely found to be the dominate PFC present in environmental samples, which is fortunate as most of the published toxicological data are on PFOS. Unfortunately, other chemicals such as PFHS, PFBS, and PFHpA were also found at elevated levels, and very little toxicological data is available on these compounds. As was shown in chapter 6.0, other PFCs whether they are short or long-chain can cause significant effects with respect to gene regulation. This could be cause for concern in

areas such as South Korea where other PFCs such as PFBS and PFHpA appear to be on the rise. The study of PFCs is relatively new and ever-changing, and as new chemicals are produced, scientists must adapt their methodologies, to not only detect these new chemicals but assess whether or not they are a risk to both humans and the environment. Future toxicological studies should focus on better understanding other PFCs commonly detected, and should not be limited to just PFOS or PFOA. Future analytical chemistry studies should focus on a wide range of PFCs, and on detecting new novel FCs.

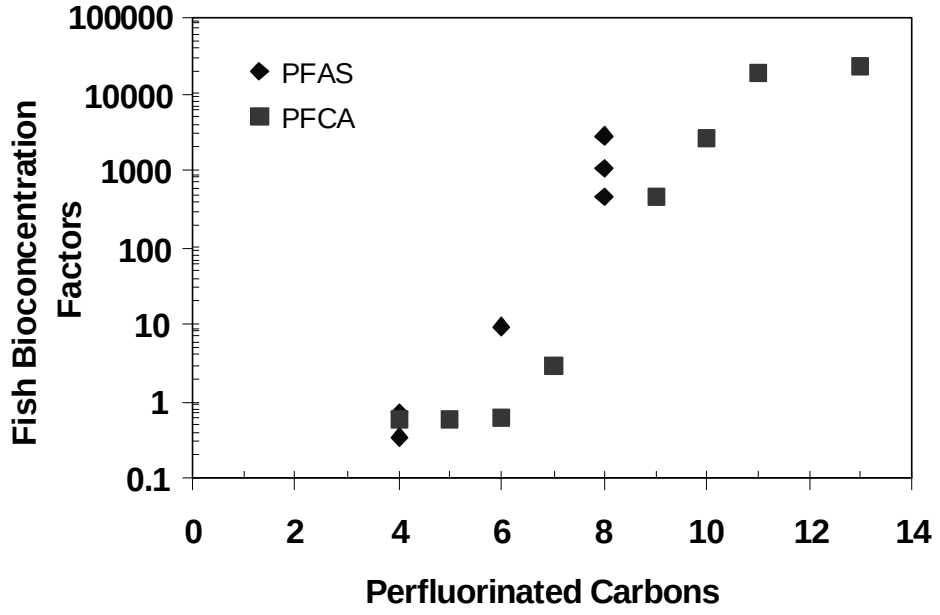
7.2 Future Directions

Future studies should focus assessing the detection and toxicological assessment of a wide variety of PFCs including recently the detected polyfluoroalkyl phosphate esters (PAPS), which have been shown to bio-transform in other more stable PFCs such as PFOA (Lee et al. 2010). This could include the analysis of archived samples to determine whether the high levels of PFCs seen in the early 2000's were in fact accurate, or whether they were the product of poor analytical methods. By re-analyzing archived samples researchers will be better able to determine the extent of PFC pollution, and whether or not concentrations are in fact decreasing as a result of the PFOS phase-out. Also researchers should continue to search for yet to be discovered PFCs and FCs, as mass balance studies have shown that only approximately 50% of the organo-fluorine in biological samples such as human blood, or marine mammal tissue is still unaccounted for (Yeung et al. 2008;Yeung et al. 2009a;Yeung et al. 2009b). By finding new organo-fluorinated chemicals and by understanding the biotransformation of some of the less stable fluorinated compounds regulators will be able to better assess any potential risk to both humans and the environment.

Few, if any studies have effectively considered the toxicity of mixtures of PFCs, which is what people and wildlife, are actually exposed to. Working with mixtures of chemicals and trying to predict their combined toxicity is always inherently difficult, but as PFCs are almost always found as a mixture of not only different chemicals, but also different isomers, studies assessing their combined toxicity are of the utmost importance. Recently (Benskin et al. 2009a;De Silva et al. 2009) have shown that exposure of different isomers of PFOS and PFOA resulted in significantly different rates of elimination, and in general the linear form being the most persistent. One rapid and effective way of helping to fill these knowledge gaps about mixtures, and PFCs other than PFOS or PFOA is by using tools such as quantitative structure activity relationships (QSARs). To this end our group recently developed QSARs to estimate the toxicity of PFCs for which no measured information was available (Giesy et al. 2010) (Figure 7.3**)**. The results of *in vitro* and *in vivo* toxicity studies with PFCs have shown that the two principle determinants of biological activity and bioaccumulation are (1) the length of the fluorinated carbon chain, and (2) the functionality of the head group (Goecke-Flora and Reo 1996;Hu et al. 2002;Lau et al. 2007). Specifically, the results of these studies have shown that the potential of PFCs to bioaccumulated is proportional to the length (number of carbons) of the fluorinated carbon chain, and that, in general, compounds that have sulfonic acid moieties tend to be more toxic than their carboxylic acid counterparts. In addition, the presences of primary and secondary amides have a significant effect on the toxicity of these compounds (Starkov and Wallace 2002). These findings can also be extended to aquatic organisms, where chain length, head group functionality, as well as the presence of amide groups, can also influence the toxicity and bioaccumulation potential of FCs.

Fluorinated compounds are an extremely useful class of chemicals, and because of their usefulness, they will continued to be used for many years to come. While these chemical are not among the toxic, their widespread distribution and steep dose-response curves are a cause for concern. Therefore it is up to regulators and industry to minimize the risks associated with the use of these chemicals. As with all chemicals, there is a need for balance between using them to improve our lives, while at the same time ensuring the safety to both the users, and the environment. The shift to shorter chain-length compounds is a step in the right direction, because these compounds impart many of the same useful chemical properties as the longer counterparts, while being less toxic and bioaccumulative. However, more toxicological research is needed to ensure that these shorter chain-length compounds are in fact less toxic to both humans and the environment.

Figure 7.3 Relationship between fluorinated carbon chain length of perfluorinated sulfonates (PFAS) and perfluorinated carboxylates (PFCA), and bioconcentration in several fish species including rainbow trout, fathead minnow, and bluegill



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